Blood Grouping TECHNIC

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FRITZ SCHIFF, M.D.
1889–1940

Blood Grouping TECHNIC

A MANUAL

for CLINICIANS, SEROLOGISTS, ANTHROPOLOGISTS, *and*

STUDENTS *of* LEGAL *and* MILITARY MEDICINE

by

FRITZ SCHIFF, M.D.
Late Chief of the Department of Bacteriology, Beth Israel Hospital, New York, N. Y.

WILLIAM C. BOYD, Ph.D.
Associate Professor of Biochemistry, Boston University School of Medicine; Associate Member, Evans Memorial, Massachusetts Memorial Hospitals, Boston

With a foreword by
KARL LANDSTEINER
Rockefeller Institute for Medical Research

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FOREWORD

Although this volume will require no recommendation, I have gladly accepted the invitation to add a few words of introduction to the English edition of Dr. Schiff's treatise on blood group technique.

Regrettably Dr. Schiff, who came to this country not long ago where he found opportunity to resume his scientific activities, did not live to witness the full completion of this book, which in its original German edition was widely used in European countries. Dr. Schiff's research work, while it encompassed significant contributions to bacteriology and anthropology, was to a large extent devoted to the subject with which this monograph deals. To mention only the most important of his investigations, he discovered the relationship between the Forssman antigen and the blood group substance A, thus connecting two lines of serological inquiries. He made the first comprehensive studies on the group-specific substances in organs and secretions and discovered the remarkable fact that the presence of group substances in secretions is governed by a special pair of genes. Further, Dr. Schiff demonstrated for the first time that blood of group O is characterized by a special agglutinogen detectable by animal sera. He initiated the chemical study of blood group substances and found materials which can be obtained in large quantities and which lend themselves to chemical investigation. His extensive studies on the heredity of the blood groups and other blood properties, distinguished by clearness of thought and precision in execution, were influential in bringing about the adoption of blood grouping tests for medicolegal purposes.

It is a happy circumstance that Dr. Boyd was willing to undertake the task not only of translating but also of revising and extending the book, he himself having notably advanced knowledge in the field.

One may anticipate that this volume will stimulate further progress and satisfy a real need.

KARL LANDSTEINER

PREFACE

In the present book an attempt is made to give clear and concise directions for carrying out blood grouping tests, not only as a preliminary to transfusion, but also in connection with cases of disputed parentage and other forensic applications, and in anthropological studies. Indirect as well as direct methods are considered. To aid the beginner in understanding the principles underlying the procedures, a brief, but, it is hoped, adequate sketch of the theoretical bases of blood grouping is presented. In connection with transfusion a number of points, some of particular interest at the present time, are taken up which bear somewhat less directly on the subject, such as the use of preserved blood, blood substitutes, and transfusion accidents. The donor problem is discussed from several angles.

It should be mentioned that the present book, like its predecessors, makes no pretense at completeness, although it is hoped that some cognizance is taken of all important modern developments. The attempt is rather to give only the technics which are tried and true, which the authors felt they could safely recommend. The number of references to the literature, also, has been restricted to a minimum.

In view of the circumstances, it may perhaps be worth while to state briefly the way in which this book was conceived and composed. Some years ago Dr. Schiff approached me with the proposal to assist him in getting out an up-to-date book on blood grouping technic, somewhat on the lines of his well known book in German, which went through three editions (Die Technik der Blutgruppenuntersuchung, Julius Springer Verlag, 3rd edition, Berlin, 1932). I found the project one to my taste, and the book was more than half completed when Dr. Schiff's scientific work was brought to a close by his untimely death. The plan of the book is due to the senior author, as is the majority of the text. I have contributed certain sections, which the reader will readily identify, and translated and revised the text of my colleague. I fear the responsibility for any errors must be largely mine, since Dr. Schiff never had the oppor-

tunity of going over the completed manuscript. It is my belief, however, that in spite of any imperfections thus resulting, the book will be of use to all who have occasion to determine blood groups, whether for practical or theoretical ends.

William C. Boyd

Kasr Ed-Dam
Bradford, N. H
March, 1942

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THEORETICAL FOUNDATIONS

1. THEORETICAL FOUNDATIONS

1.1 Group Differences Detectable by Iso-Antibodies. (The Four Classical Landsteiner Blood Groups)

1.11. Isohemagglutination

If sedimentated red blood corpuscles are shaken up, as a rule they disperse themselves evenly, each corpuscle remaining separate. Only in special cases do the corpuscles stick to each other, with their flat surfaces in contact, like stacks of coins, forming *rouleaux* (Fig. 1*d*). If the fluid is agitated or diluted, the rouleaux disperse, and an even suspension is obtained as before.

If the red blood corpuscles of one individual are mixed in the test tube with the blood serum of another individual, they may disperse themselves as evenly here as in their own serum (Fig. 1*a*). However, this is not always the case; the corpuscles of some persons in the serum of certain other persons aggregate quickly to form clusters and clumps (Figs. 1*b* and 1*c*). This is *agglutination*, a serological phenomenon related to bacterial agglutination. Since the blood corpuscles are agglutinated, it is designated as *hem*agglutination. We speak further of *iso*hemagglutination, because the corpuscles are affected by the serum of the same species. The isohemagglutinins, of which we shall speak here, are not obtained by immunization, like bacterial agglutinins, but are constituents of normal serum.

1.12 Differentiation of Two Bloods by Direct Mixture

The phenomenon of isohemagglutination permits us to demonstrate, in some cases, that two blood samples, in spite of superficial similarity, are of unlike serological constitution, and must be of different origin. Since "autoagglutination", or clumping of blood corpuscles by their own serum, does not occur under ordinary experimental conditions, a difference between two samples of human blood generally exists if the corpuscles of one sample are agglutinated by the blood serum of the other. It is sufficient to allow the two samples to react with each other, under the proper experimental conditions (cross-matching).

1.13 Differentiation of Human Bloods by Classification into Groups

Landsteiner (14) showed that the isoagglutination phenomenon obeys certain rules, and under some circumstances this permits differentiation of two blood samples without mixing them. Every human blood can,

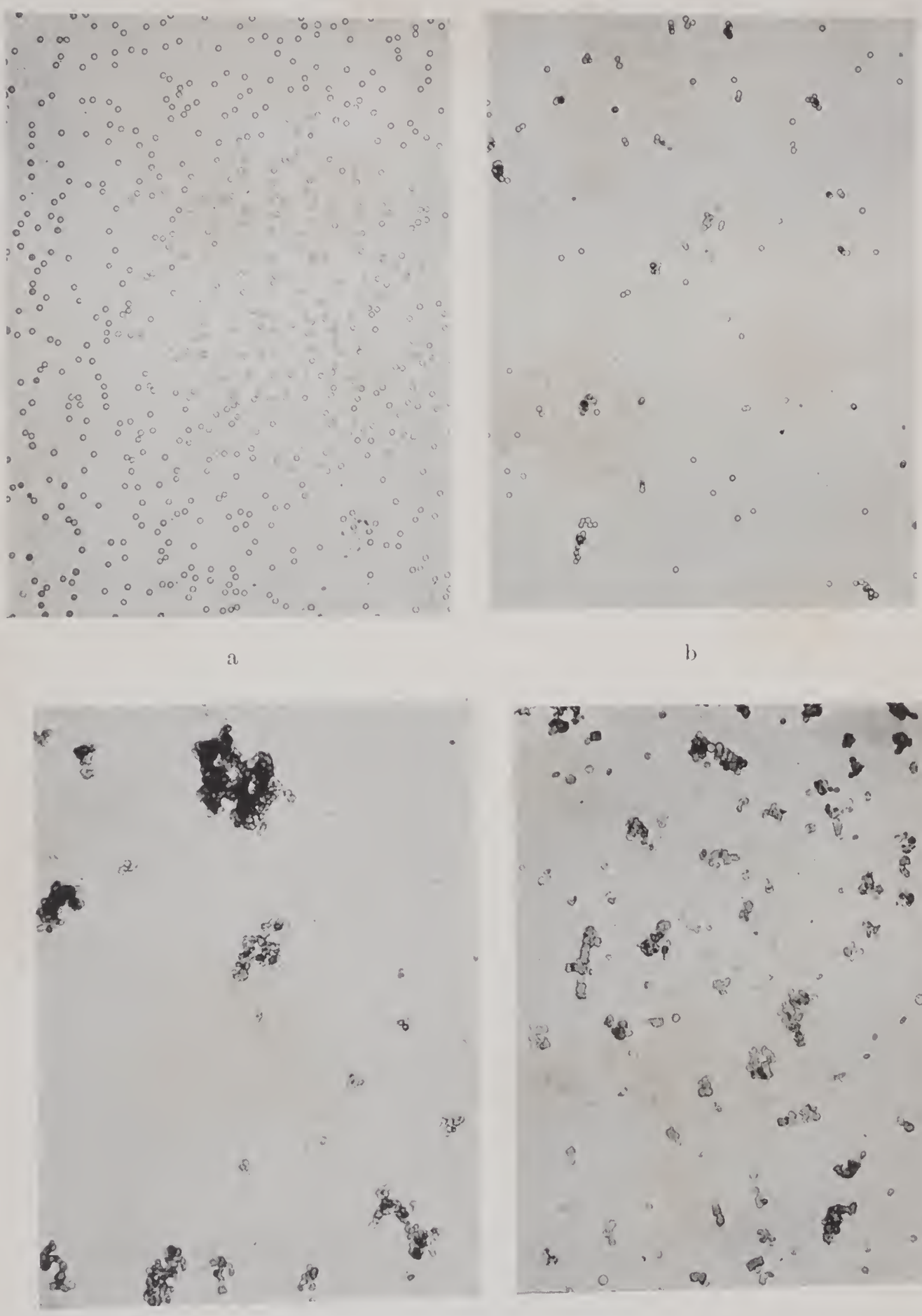

Fig. 1. Photographs of hemagglutination and rouleaux: (a), negative reaction; (b), weak reaction; (c), strong reaction; (d), rouleaux.

according to its behavior in isohemagglutination, be classified in one of four groups, the *blood groups*.

A difference in blood group denotes unlike serological constitution; in this case agglutination would occur on cross-matching. Identity in blood group implies similar behavior in the isoagglutination test, and absence of agglutination on cross-matching. Blood samples of different group must always have come from different persons, but samples of the same group do not necessarily come from the same person, of course.

1.14 Basis of Division into Groups

The division of all persons into four blood groups depends upon the fact that there are two different blood corpuscle characteristics, or isoagglutinable substances, designated by the letters A and B. They can be present singly or together, or can be absent. If we designate the absence of both by O, we have four possibilities:

1. Blood corpuscles contain O
2. Blood corpuscles contain A
3. Blood corpuscles contain B
4. Blood corpuscles contain A plus B

These four possibilities correspond to the four blood groups which are now, by international agreement, designated briefly by the above mentioned letters, thus:

O A B AB

In peoples of European stock, groups O and A are the commonest, running about 40% each. The blood groups B and AB represent about 10–15% and 5% respectively of the population (cf. sec. 3.62).

The blood group O is characterized by the lack of sensitivity of its corpuscles to any of the isoagglutinins. The other blood groups possess agglutinable blood corpuscles. The symbol A implies the reactivity of the corpuscles with a certain serum agglutinin which is designated as anti-A or α. It will be seen that this reactivity is found in *two* blood groups, group A and group AB.

Correspondingly, corpuscles which possess B (group B and group AB) react with the anti-B or β of sera which contain these agglutinins.

The relation of the serum agglutinins anti-A and anti-B to the characteristic blood corpuscle substances of the individual is given by the *Landsteiner rule*: there is always found that agglutinin or agglutinins which could coexist physiologically with the blood group characteristic which is present. Thus, for example, anti-A is found in the presence of O and B, but not of A. These relations are illustrated by the following scheme (Fig. 2), and by Table 1, in the last column of which the blood group characteristics are all represented together.

1.141 Nomenclature of Groups

Two systems of designating the blood groups by numbers were formerly in use, and are still to be met with in certain hospitals. They were proposed by Jansky and by Moss. The classification of Moss unfortunately

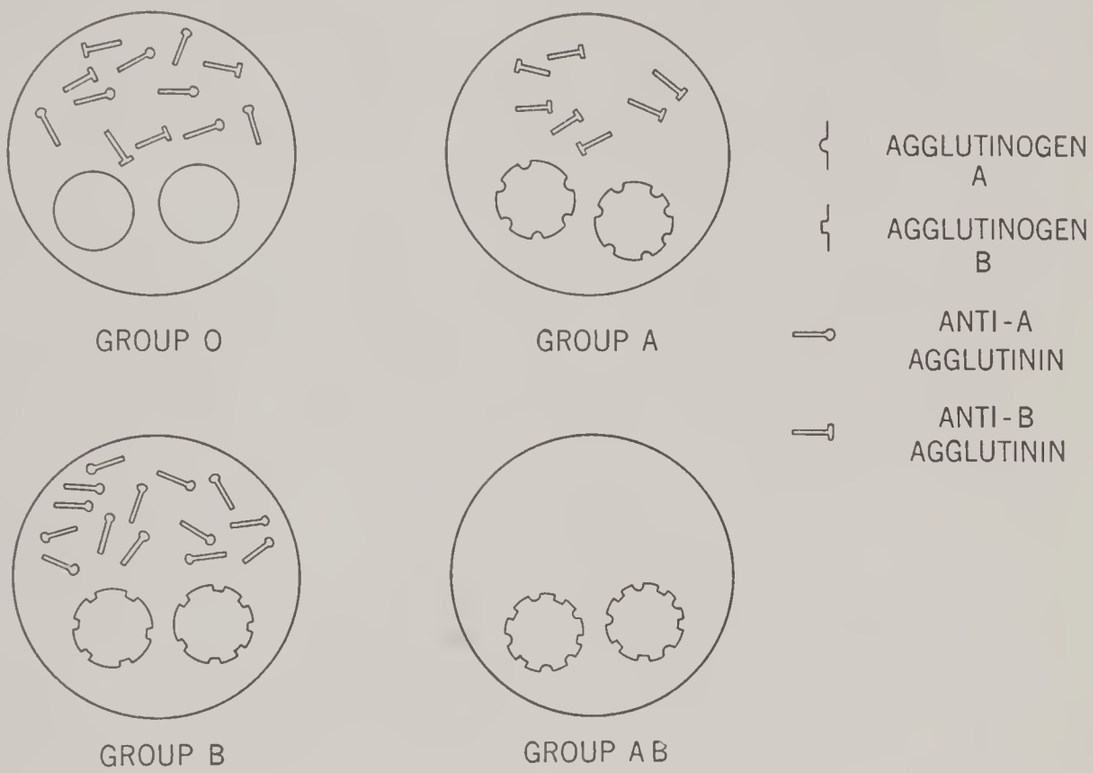

Fig. 2. Diagrammatic representation of cell receptors and agglutinins in the four groups.

TABLE 1

Group			Blood corpuscles		Serum	Group formula
International	Jansky	Moss	Contain the agglutinable substance (agglutinogen)	Are agglutinated by serum of group	Contains agglutinin	
O	I	IV	O		anti-A + anti- B	O (anti-A, anti-B)
A	II	II	A	O + B	anti-B	A (anti-B)
B	III	III	B	O + A	anti-A	B (anti-A)
AB	IV	I	A + B	O + A + B		AB (o)*

* o = simultaneous absence of agglutinins anti-A and anti-B.

reversed the significance attached to the symbols I and IV by Jansky, and this has led to much confusion. For all scientific purposes these systems have now been replaced by the internationally adopted letter system, which goes back to a proposal of von Dungern and Hirszfeld. The relations of the three nomenclatures can be seen from Table 1.

In hospitals where either of the older nomenclatures is in use, it is

important that they be replaced by the international system to avoid confusion and possible accidents.

According to the proposal of the Division of Hygiene of the League of Nations, during the time of transition the designation formerly used should be written in parentheses. Thus, one would write:

Jansky	O (I)	A (II)	B (III)	AB (IV)
Moss	O (IV)	A (II)	B (III)	AB (I)

For test sera, the Committee requires that the agglutinins be mentioned expressly, in addition to the group designation, thus:

test serum A (anti-B)
test serum B (anti-A)

1.15 Basic Rules for Group Determination

Any given blood can be grouped by two different, independent methods. One can test the unknown blood corpuscles with two sera, anti-A and anti-B, or the unknown serum can be allowed to act on known corpuscles A and B. In special cases a combination of the two methods can be used. One can use a test serum and corpuscles of the same group (or even from the same person) provided serum and corpuscles both belong either to group A or group B.

TABLE 2

DETERMINATION OF GROUPS WITH TWO TEST SERA, ANTI-A AND ANTI-B

	Known serum anti-A (group B)	Known serum anti-B (group A)	Group
Agglutination of the unknown blood corpuscles	−	−	O
	+	−	A
	−	+	B
	+	+	AB

1.151 Using Known Sera

The preparation of agglutinating sera of types anti-A and anti-B is described below. These are mixed with the unknown corpuscles. According to whether the corpuscles are agglutinated by neither, or one or the other, or both of the unknown test sera, we have four possibilities which correspond to the four blood groups; see table 2.

1.152 Using Known Blood Corpuscles

The known corpuscles A and B are mixed with the serum to be investigated. Here also four possibilities arise and each one of them corresponds to a certain blood group (table 3).

1.153 Using Corpuscles and Serum of a Single Known Group

The details are shown in Tables 4 and 5.

TABLE 3

DETERMINATION OF GROUPS WITH TWO KINDS OF BLOOD CORPUSCLES, A AND B

	Known corpuscles A	Known corpuscles B	Group
Agglutination by the unknown serum	+	+	O
	−	+	A
	+	−	B
	−	−	AB

TABLE 4

BLOOD GROUP DETERMINATION USING CORPUSCLES AND SERUM OF GROUP A (ANTI-B)

	Known serum A (anti-B)	Known corpuscles A	Group
	Unknown corpuscles	Unknown serum	
Agglutination	−	+	O
	−	−	A
	+	+	B
	+	−	AB

TABLE 5

BLOOD GROUP DETERMINATION USING CORPUSCLES AND SERUM OF GROUP B (ANTI-A)

	Known serum B (anti-A)	Known corpuscles B	Group
	Unknown corpuscles	Unknown serum	
Agglutination	−	+	O
	+	+	A
	−	−	B
	+	−	AB

1.16 Permanence of the Blood Groups

The blood corpuscle properties which are used for the determination of the blood group are already established in the new-born and in the fetus. On the other hand, the child's characteristic serum properties are not demonstrable at birth. They develop in the first months of life, or in any case are fully established at the end of two years.

A change in blood group has never been definitely established. Observations to the contrary, for example, apparent influence of medicaments, X-rays, electricity, etc. on blood groups, without exception arise from inadequate technic, and in fact the necessity of reliable technic is never better illustrated than by the literature dealing with the alleged "changes" of blood group.

The observation of temporary irregular agglutinins following blood transfusion naturally does not contradict the rule that the blood *group* of an individual is unalterable. The same applies to changes in the agglutinability of the cells, as observed for instance by Levine and Katzin (21).

1.17 Inheritance of the Blood Groups

The blood groups are inherited according to the Mendelian law, the corpuscle characteristics A and B not appearing in the child unless present in at least one of the parents. A and B thus behave as strict dominants (4). The exact mechanism is explained in detail in sec. 3.33. Absence of A and B may be indicated by "O".

Since each body cell has a pair of chromosomes, each of which carries or fails to carry one of these factors, an individual's genetic constitution may be represented by AB, AA, AO, BB, BO, or OO, where O represents the absence in the chromosome of either the A or B factor at that point. The fact that the AO individual has only one dose, so to speak, of the A factor makes no practical difference, and his blood is routinely indistinguishable by direct test from that of the AA individual. This means that we have but four types of blood (phenotypes), O, A, B, and AB, ordinarily distinguishable. (See Figure 3.)

Genetically, the four blood groups correspond to four different phenotypes. The corresponding genotypes can be seen from the theory of inheritance proposed in 1924 by Bernstein (2). According to Bernstein, to each blood group characteristic, O, A, or B, there corresponds a certain gene. These genes, designated as O, A, and B, are located at the same point of a certain chromosome and mutually exclude each other from this place (unilocal or multiple allelomorph factors). O ("absence of A and B") is recessive to A and to B. Between A and to B there is no definite dominance. The blood group characteristics A and B are recognizable in the presence of each other.

1.2 The Sub-Divisions of the A Characteristic

1.21 A_1 and A_2 (Landsteiner and Levine)

In the case of the characteristic A, two sub-types, A_1 and A_2, called in German "A-gross" and "A-klein" were distinguished (5). They can be demonstrated either by certain irregular isoagglutinins of normal human sera, specific for A_1 or A_2, or with suitable technic by the ordinary group agglutinin anti-A. Blood of type A_1 absorbs the agglutinin anti-A more

strongly than does A_2. To absorb definite amounts of agglutinin, less A_1 is sufficient than A_2. Group AB can be similarly subdivided. The technic of differentiating the subgroups is discussed in sec. 2.52.

The sub-group A_1 is about five or six times as common as A_2. The subgroups are determined by inheritance. Thomsen and co-workers assume a dominant gene A_1 and a recessive gene A_2. In the series O, A, B of multiple allelomorphs, A can be subdivided into A_1 and A_2, and we have the four multiple allelomorphs, O, A_2, A_1, B. This scheme seems to be followed regularly, or almost regularly.

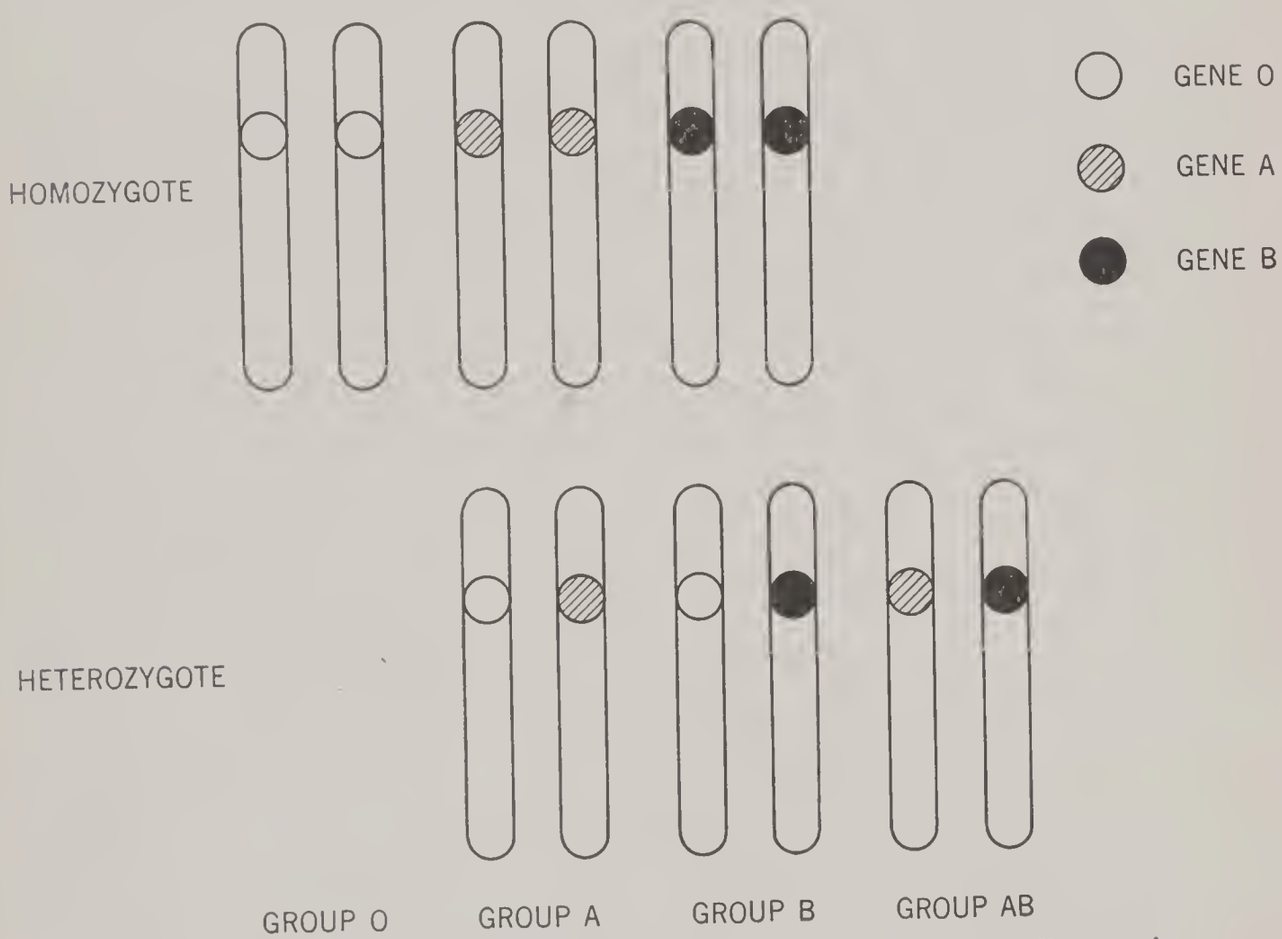

Fig. 3. Genetic formulas of the blood groups according to Bernstein

Later workers have found still further sub-divisions of the A characteristic.

1.22 A_3 and A_4

Fischer and Hahn (6), and Friedenreich (7) reported the existence of a third sub-group of group A, which was designated as A_3. It is agglutinated by B sera even more weakly than is A_2. It seems to be very rare (perhaps one out of 2000 persons). Gammelgaard and Marcussen (12) have recently reported the existence of still a fourth allelomorphic gene of A, designated as A_4. It is agglutinated even less readily than A_3; immune

rabbit sera and certain O sera were found best for its detection. One example was found in 60,000 routine examinations. (See sec. 2.525.)

1.3 The M, N Types

1.31 General

The A, B groups do not exhaust the list of individual differences in human blood, but they are the only ones which can be regularly demonstrated without other reagents in addition to normal human blood. Even the A, B groups can be demonstrated quite readily with the aid of immune agglutinins produced by injecting rabbits with human blood, and the same method led Landsteiner and Levine (15, 16) to the discovery of the human blood factors M and N.

The production of antisera specific for M and N will be discussed below, sec. 2.4411. Three different types of human blood can be distinguished with their aid: bloods containing M only, bloods containing N only, and bloods containing both. No blood having neither M nor N has ever been found. These M, N types are quite independent of the A, B groups, just as men might be classified into tall and short, and independently into light-haired and dark-haired.

1.32 Inheritance

The M, N types are inherited independently of the four classical A, B groups. Their inheritance depends upon a single pair of hereditary units (genes), which we may designate as M and N. Since the inheritance is independent of that of A and B, the two sets of genes evidently must lie on different chromosomes. The way the three types (phenotypes) are determined by the three possible combinations of the two genes M and N is shown in Table 6.

1.33 Subgroups of M and N; M_2 and N_2

More weakly agglutinable forms of the agglutinogen N have been reported (cf. 8). They are of rare occurrence. One case of an M factor reacting more weakly with about half the anti-M sera, but more strongly with about one-fourth of them, has been reported (10). For the technic of distinguishing N_1 and N_2, see sec. 2.54.

1.4 Other Individual Differences Detectable Serologically

1.41 General

In addition to M and N, a number of other individual blood differences detectable serologically have been reported. A summary is shown in Table 7. Of these, the factors P and Rh have at the present time the most importance (secs. 2.61 and 2.62).

TABLE 6

GENETIC CONSTITUTION OF THE THREE DIFFERENT M, N TYPES OF BLOOD

Type	Genetic constitution
M	MM
MN	MN
N	NN

TABLE 7

OTHER AGGLUTINABLE FACTORS REPORTED IN HUMAN ERYTHROCYTES (IN ADDITION TO A, B, M, AND N)

Factor	Investigators	Date	Demonstrated with	Chief properties
P	Landsteiner and Levine (17)	1928	Immune rabbit serum	Graded reactions; more frequent in negroes than in whites
G	Schiff (25)	1932	Immune rabbit serum	In most A's and B's; O may absorb but is never agglutinated
H	Schiff (25)	1932	Immune sheep serum	Related to P?
Hunter factor	Landsteiner, Strutton and Chase (18)	1934	Immune rabbit serum	Rare in whites, far more frequent in negroes
X	Andresen (1)	1934	Immune rabbit serum	Related to P?
Q	Furuhata and Imamura (11)	1935	Pig serum	Q dominant over non-Q; related to or identical with P
E	Sugishita (29)	1935	Eel serum	
Rh	Landsteiner and Wiener (19)	1940	Immune rabbit serum	Common to human and rhesus blood; Rh+ more common than Rh−
"	Landsteiner and Wiener (20)	1941	"Guinea pig"	
"	Wiener and Peters (30)	1940	Post-transfusion human serum	
"	Levine and co-workers	1940	Serum from mothers of infants with erythroblastosis fetalis	

1.42 The Number of Different Types of Blood Distinguishable

It will readily be appreciated that by the simultaneous application of tests for all of the factors reported in human blood corpuscles, a large number of different types could be distinguished. If we limit ourselves to those

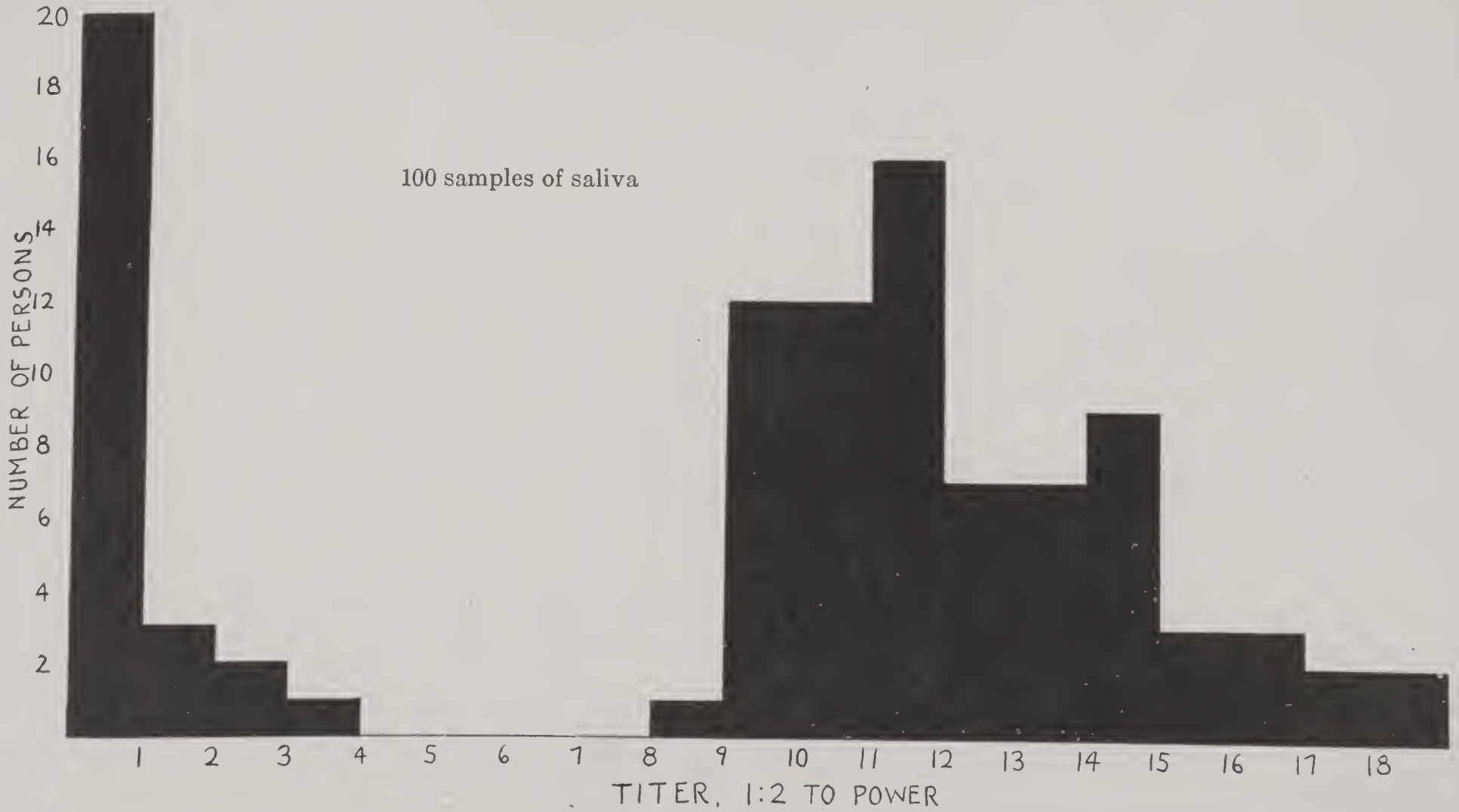

Fig. 4. Amounts of group antigen in the salivas of 100 individuals of groups A and B (Hartmann).

which have been most studied, and for which sera are currently available, we have the following possibilities:

A, B system:	O, A_1, A_2, A_3*, B, A_1B, A_2B, A_3B*	(8 classes)
M, N system:	M, MN_1, MN_2*, N_1, N_2**	(5 classes)
P system:	P +, P −	(2 classes)
Rh system:	Rh +, Rh −	(2 classes)

$8 \times 5 \times 2 \times 2 = 160$ kinds of human blood distinguishable serologically.

1.43 The "Secreting Factor"; Secreting and Non-Secreting Types

Antigens A and B are not invariably present in the saliva when present in the blood. If the content of group substance in the saliva is examined, it will be found that individuals fall into two well-defined classes, designated as secretors (German, Ausscheider) and non-secretors (Nichtausscheider), (see Fig. 4). This difference is hereditary, and seems to be determined by a pair of Mendelian genes, designated by S (secretor) and s (non-secretor) (27, 28). The secreting type is the more common. The relative proportion of secretors and non-secretors seems to vary in different races. There is evidence that the group substance found in the saliva is actually manufactured in the salivary glands (9).

Table 8

Inheritance of "Secreting" Factor

Mating	Number of families	Children	
		S	s
S x S	51	134	21
S x s	38	64	44
s x s	11	0	28

S = secretor, s = non-secretor. Data from Schiff and Sasaki (27, 28), Matson and Brady (23), and Zieve, Wiener and Fries (31).

Schiff was able to separate persons of group O into secretors and non-secretors by means of anti-O serum, such as cow serum absorbed with AB, and/or a heterogenetic serum (serum of a goat injected with Shiga bacillus) one of which reacted more strongly with O than with other groups, (26).

Contrary to what is observed in secretors, in the organs of non-secretors little or no water-soluble A or B substance is found (9, 13). The group substances are nevertheless present, can be extracted by alcohol (13),

* Very rare (see above).

** Has never been described.

and the group specificity of organs of non-secretors can be demonstrated by absorption experiments (24, 3).

Some animals excrete in their saliva (and perhaps other fluids) substances related serologically to the A and B structures of man. (See sec. 3.51).

References

In addition to the specific citations given below, which are referred to in the text of this chapter, the reader will find further information of a general nature in well-known text books and monographs on this subject, of which we may mention a few:

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* (N.V.) = reference not verified.

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GENERAL TECHNIC OF BLOOD GROUP INVESTIGATION

2. GENERAL TECHNIC OF BLOOD GROUP INVESTIGATION

2.1 Apparatus

A good electric centrifuge and an electric refrigerator or cold room are perhaps the most important and essential features of a blood grouping laboratory. A water bath also is sometimes useful. The most generally useful type of centrifuge for blood work is the Size 2 centrifuge manufactured by the International Equipment Company, for it can take care of very small as well as fairly large quantities. Cups plus tubes should be carefully balanced, and balanced pairs placed opposite each other in the machine. Centrifuge tubes holding about 100 cc., made of Pyrex glass, should be available, with a head and cups of suitable size. For smaller quantities test tubes of the "Wassermann" type, or smaller, may be used, in smaller cups interchangeable with the larger ones.

Test tubes of the "Wassermann" type, roughly 100 x 13 mm., holding 8–9 cc., are suitable for a large number of tests. Occasionally larger tubes may be needed. For blood grouping tests, tubes measuring about 75 x 9 mm., holding about 4 cc., and without lip, are desirable. It is convenient to have two kinds which are distinguished by colored markings (red and green bands at the mouth). Five of these tubes may be placed in a 60 cc. centrifuge cup. It is convenient to have racks on hand in which to put the tubes (Fig. 5). So-called Kolmer racks are suitable.

For the performance of precipitin tests, "micro" tubes (Fig. 32) specially made from semi-capillary tubing are desirable to avoid waste of material. Centrifuge tubes holding 50 and 100 cc. are useful. Occasionally graduated centrifuge tubes, holding about 15 cc., will be found useful. It is desirable if possible to have all test tubes made of Pyrex glass.

Test tube racks, preferably of copper, adapted to holding Wassermann tubes, will be desirable. The size may depend on individual preference; a type holding two rows of 6 or 8 tubes each will be found useful. For field work in blood-grouping, galvanized wire racks of the Kolmer type, holding 4 rows of 12 each, are better. For some of the smaller types of tubes it may be necessary to make suitable racks or supports. For larger tubes, such as the large centrifuge tubes, galvanized wire baskets, preferably of the square or rectangular type, are useful.

Several types of pipettes will be needed. Some of the ordinary chemical volumetric pipettes, in sizes of 5, 10, and 20 cc. will be useful, but in addition pipettes of the serological type (graduated to the tip) in sizes of

1, 2, 5, 10, and 15 cc. will be wanted. For certain kinds of work other types, for instance a 0.2 cc. pipette graduated in 1/100ths may be desired. So-called capillary pipettes, made by drawing pieces of ordinary (7–8 mm.) laboratory glass tubing out to a fine (ca. 1 mm.) capillary, used with rubber bulbs, will be almost indispensable. These are harder to make if Pyrex tubing is used, but the resulting pipettes are much more durable.

For many kinds of work sterile pipettes are needed. Before sterilization cotton is stuffed into the upper end of the pipette, not too tightly, and the excess burned off by quick passage through a flame. They are

Fig. 5. Test tube rack and tubes for blood grouping, showing use of capillary pipette (*photograph by Dr. H. K. Giffen*).

sterilized in the hot air oven in copper cans, preferably of the rectangular type, each size in a separate can.

For sterilization of serum, where heating would be deleterious and it is not desired to add a preservative, filters of the Berkefeld or Mandler type will be needed. These are used in connection with a sterile side arm filter flask (of Pyrex) holding about 250 cc. A water or mechanical pump to provide suction is almost essential. (See however, (9).)

As a preservative of biological materials, 'merthiolate' (Lilly) in a concentration of about 1:5000 is good. For preserving as well as marking blood-grouping sera, certain dyes (acriflavine, brilliant green) have been found good (71, 4). According to Conn (14), brilliant green is the same

as Ethyl Green and Malachite Green G. Its C. I. number is given as No. 662. The preparation we used was brilliant green (Nat. Aniline Co.), Schultz No. 499, Cert. No. NBg-1. The acriflavine was a preparation of the Heyl Laboratories, N. Y. and Chicago. According to Conn (14, p. 141), the C. I. number is 790. Another preparation of neutral acriflavine was found to be only partly soluble and apparently less effective.

For the preservation of erythrocytes for agglutination tests, the mixture of Rous and Turner (72, 42) (5 vol. 5.4% glucose, 2 vol. 3.8% sodium citrate, 3 vol. blood) is very suitable. For material which is to be injected, phenol to 0.5 per cent has been found satisfactory.

The cleaning of glassware is much easier if it is immersed in water immediately after it has been used. When infectious material has been used pipettes may be placed in one per cent formalin. When pipettes become greasy inside, ordinary washing is not sufficient, and they should be soaked 24–48 hours in a solution of sulphuric acid and dichromate, for instance,

Fig. 6. Spring blood lancet (*courtesy of Progressive Laboratory Specialties Company*)

100 g. $K_2Cr_2O_7$, 1 L. H_2SO_4, 200 cc. H_2O. Cloudy glassware should never be used, unless it is ascertained (by treatment with this cleaning solution) that the cloudiness is due to etching of the glass.

Two types of syringe, the Luer all-glass (Pyrex) in sizes of 5, 10, 20, or 30, and 50 cc., and the "tuberculin" 1 cc. syringe, and needles of various sizes, will be needed.

For injecting or bleeding guinea pigs, a holder consisting of a board with numerous holes in it, plus a carrier holding a cross bar for the head, is useful. The animal's legs are tied down with loops of rawhide. This holder is described and illustrated in (11).

For rabbits, various holders have been recommended, from a plain towel to a box with a sliding cover which has a notch for the neck, in which the animal is placed with the head outside (11). We have found it perfectly feasible, and on the whole simpler, to hold the rabbit on the lap (which is protected by a towel or thick laboratory coat) while bleeding from the ear or injecting into the ear veins.

For taking blood from the ear lobe or finger of patients, a spring blood lancet is very useful (see figure 6). An ordinary needle can be used.

An ordinary microscope, with slides and cover slips, will be found of assistance, but is not indispensable. "Well-slides", which can be made by putting paraffin or bone rings on glass, or glass or porcelain slabs with depressions, are useful in some technics (see for example, sec. 3.6533).

2.2 Isohemagglutination.

2.21 Selection of A and B Test Bloods.

2.211 Initial Search for Test[1] Bloods

Individuals of known blood group are to be found in most laboratories. If blood of known group is not available, it is necessary to locate bloods of groups A and B.

2.2111 Principle. Blood of groups A and B is needed. Two blood samples belong to these groups when each serum agglutinates the corpuscles of the other sample. With two such samples available, it is still unknown

Table 9

Agglutination Resulting from "Cross Testing" 12 Bloods

Blood sera	Blood corpuscles												
	1	2	3	4	5	6	7	8	9	10	11	12	
1	−	+	+	+	−	−	+	−	−	+	+	+	O
2	−	−	−	−	−	−	−	−	−	+	−	−	A
3	−	−	−	−	−	−	−	−	−	+	−	−	A
4	−	−	−	−	−	−	−	−	−	+	−	−	A
5	−	+	+	+	−	−	+	−	−	+	+	+	O
6	−	+	+	+	−	−	+	−	−	+	+	+	O
7	−	−	−	−	−	−	−	−	−	+	−	−	A
8	−	+	+	+	−	−	+	−	−	+	+	+	O
9	−	+	+	+	−	−	+	−	−	+	+	+	O
10	−	+	+	+	−	−	+	−	−	−	+	+	B
11	−	−	−	−	−	−	−	−	−	+	−	−	A
12	−	−	−	−	−	−	−	−	−	+	−	−	A
	O	A	A	A	O	O	A	O	O	B	A	A	

which is group A and which is group B. The absolute identification of the two groups depends upon having known samples available. However, orientation is generally possible from the fact that group A is commoner in America and in Middle and Western Europe than is group B.

If a number of blood samples are tested for their mutual relationship, the serum of each person being tested with the corpuscles of each other person ("cross-matching", see sec. 1.12), in many of the mixtures agglutination appears quickly, and in others it remains absent.

2.2112 Procedure. If we start with 12 blood samples, for example, such an experiment may take the following form (table 9).

Consider first the vertical columns which give the behavior of the corpuscles. The corpuscles of groups A and B, which we are searching

[1] The designation "test blood" is used in the following for serum and for corpuscles of known groups.

for, contain agglutinable substances. Since the corpuscles in columns 1, 5, 6, 8, and 9 are not agglutinated, they evidently belong to group 0 and can be left out of consideration.

Among the remaining columns, we at once recognize a separation into groups. The vertical columns 2, 3, 4, 7, 11 and 12 behave alike. The corpuscles of column 10 behave differently. So we may begin with the designation of type "2, 3, 4, etc.," and type "10".

On the basis of the known frequencies of the blood groups (compare above p. 5), we assume that the type "2, 3, 4 etc." corresponds to group A and type "10" corresponds to group B. If this assumption is correct, these sera should contain the proper agglutinins. The horizontal rows show that both type "2, 3, 4, etc." and type "10" do in fact possess agglutinins which correspond to the scheme, and agglutinate the opposite type of corpuscles. Next we must verify that type "2, 3, 4, etc." is really the commoner. For this purpose, we mix the corpuscles of about ten persons with the serum of "2"* and with serum "10". All persons whose corpuscles are agglutinated by serum "10" and not by serum "2" belong to the same group as "2", and those which react with serum "2, 3, etc." and not with "10" belong to the same group as "10". We might find three samples belonging to the same group as "2, 3, etc." and two more of the other type. Then we should have altogether nine samples of type "2" and only three of type "10". Therefore "2" is definitely more common than "10" and the type "2" corresponds to group A and type "10" to group B. In Western Europe or America, a definite decision could certainly be made by the investigation of 25 or 30 persons. In regions where the frequency of the blood groups is unknown, a known blood A or B must be available for comparison.

* Or we could use another serum of the same type, or a mixture of the sera "2, 3, 4, 7, 11, and 12".

2.212 Listing Standard Bloods

Once test sera anti-A and anti-B are available, determine the blood groups of your immediate associates in the laboratory. In this way standard donors for experimental purposes are obtained to whom one can have recourse in case of necessity.

2.2121 Economy in Use of B Serum. If only one or two individuals of group B should be found, and the test serum B (anti-A) is hard to obtain, subsequent tests may be set up with serum O (anti-A, anti-B) and serum A (anti-B). All tests which react negatively with serum A (anti-B) and at the same time react positively with serum O (anti-AB) belong to group A. Those reacting negatively with O serum belong to group O. In this way, the group of about 80% of all samples could be

determined without the use of serum B, and this serum can be used merely for the remaining 20%, i.e., those blood samples reacting with both serum O and serum A. These bloods belong to groups B and AB, and may be distinguished by the fact that in the first case the B serum (anti-A) will give a negative reaction, and in the latter case, a positive reaction. Table 10 will make these relationships clear.

2.22 Selection of Most Suitable Test Blood

Different sera of the same group do not all react equally strongly; there is a good deal of difference in content of agglutinin. This is most evident if the sera are successively diluted and the dilution of each serum which still just agglutinates is determined (limiting titer). The strength of sera and the differences in the degree of activity are shown in figure 7 (from (35)). Here the limiting titer of 100 anti-A sera is shown, determined

TABLE 10

DETERMINATION OF BLOOD GROUPS O AND A WITH TEST SERUM O (ANTI-A, B) AND A (ANTI-B)

	Known serum		Blood group
	O (anti-A, B)	A (anti-B)	
Agglutination of unknown blood corpuscles	−	(−)[1]	O
	+	−	A
	+	+	B or AB

[1] Not required.

by tests with the corpuscles of a single person. Serum is ordinarily used straight, or only slightly diluted, so that generally a considerable multiple of the minimal active amount of agglutinin is used. Thus even weaker sera may be found to act promptly and fairly strongly with most bloods.

There are also differences in sensitivity between the corpuscles of different persons. Figure 8 illustrates these. The curve gives the limiting titer of the same serum against the blood corpuscles of different persons. If all the blood samples are investigated the day they are taken, relatively small variations in the sensitivity are found; the graph, which combines fresh samples and samples a few days old, corresponds to the results found in laboratory practice.

In view of these facts, even when a test blood A and a test blood B have been found, they will not be used as reagents without further trial, save in exceptional cases. It is better to choose especially strongly active sera and especially sensitive corpuscles.

2.221 Selection of Strong Test Sera. The Serum Donor

It is convenient to have persons with known blood groups and strongly agglutinating sera available, so that fresh suitable sera may be obtained

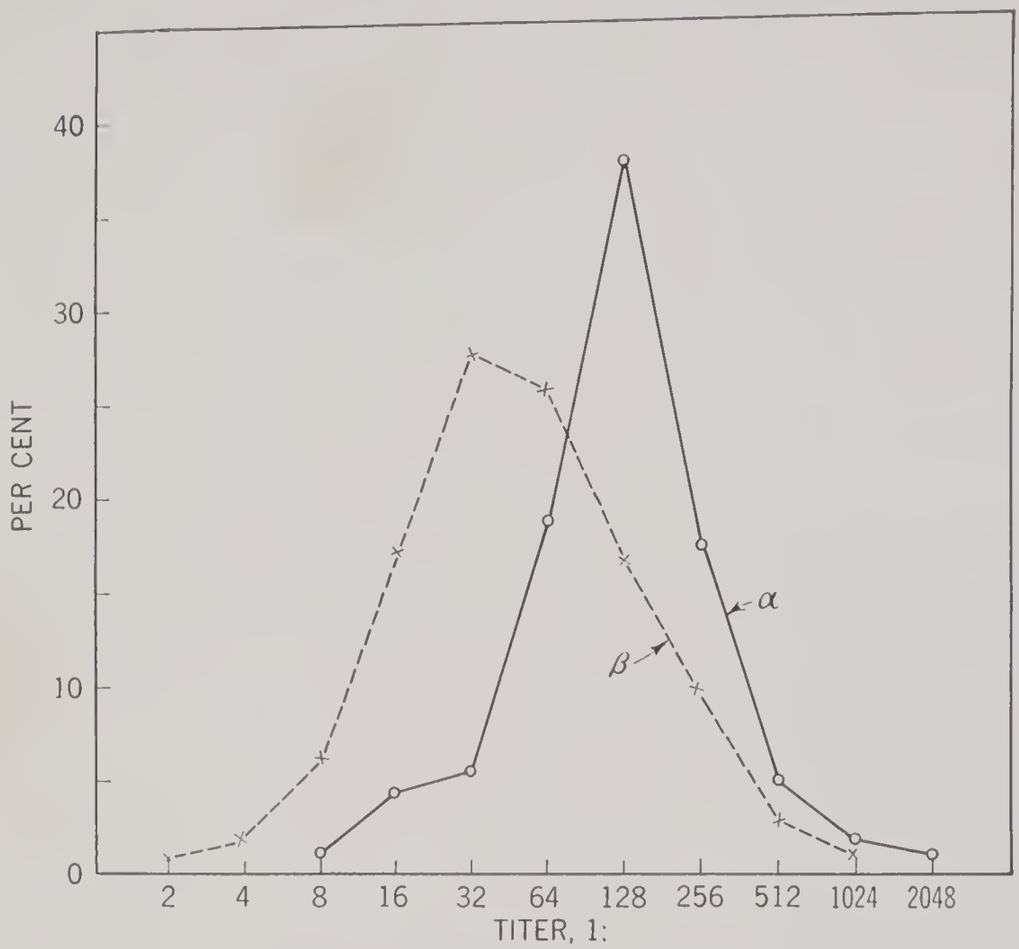

Fig. 7. Titers of sera from different persons

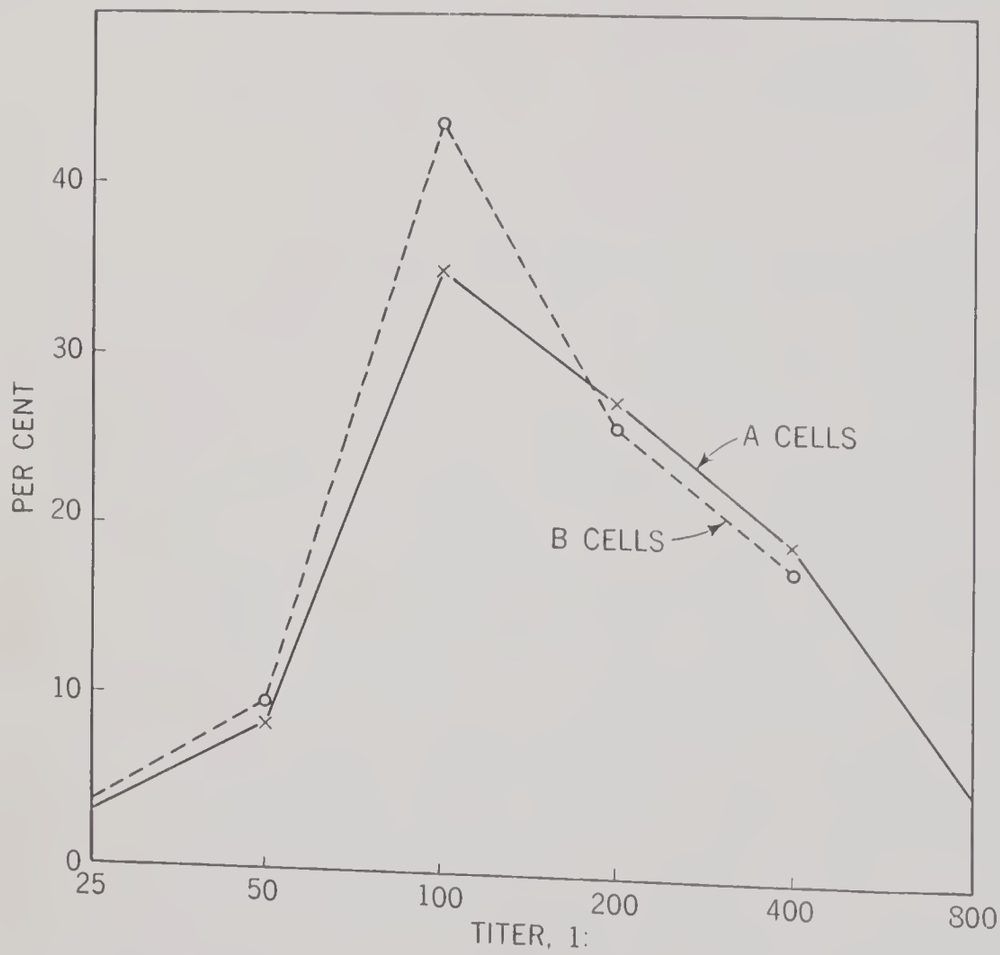

Fig. 8. Sensitivity of cells from different persons

when needed without special tests. The serum of adults who are not too old is strongest. In early youth, as well as in great age, the agglutinins are often weaker (figure 12).

In general, the blood of healthy persons is to be preferred to that of those who are ill. With certain exceptions, however, the sera of patients, such as those coming up for Wassermann tests, are generally usable. Care is needed in the case of diseases in which the sedimentation velocity of the erythrocytes is pathologically increased, as with the blood of patients with carcinoma, high fever, and women during pregnancy and confinement. Cord blood is completely unsuited for test blood. On account of the danger of infection, blood samples which have been sent in for the Widal test should not be used.

Fig. 9. Ring maker for making well slides (*courtesy of Progressive Laboratory Specialties Company*).

2.2211 Estimation of Strength of Sera. For routine use the strongest sera should be selected from a number available in sufficient quantity. Since the average strength of sera is low, it is not advisable to pool a number of sera all of the same group, merely taken at random.

Two procedures should be employed to test the suitability of grouping sera: determination of limiting titer, and estimation of the strength and speed of agglutination with standard cells. The serum is diluted with saline 1:10, then successively 1:20, 1:40 etc., and tested by the test-tube-centrifuge technic against standard erythrocytes. A serum to be suitable should show a strong reaction at least in a dilution of 1:10. In making the dilutions, pipettes with cotton in the end should be used, to prevent possible entrance of saliva, which may contain inhibiting substances.

The limiting titer should not be relied on alone, for some sera apparently can stand high dilution, but give, even when concentrated, rather weak agglutination (low avidity agglutinins). Therefore each serum should be tested also on a slide. A mixture of one drop of serum and one of cell suspension should give, with thorough mixing, definite agglutination visible to the naked eye in one minute at the latest.

Coca (12) proposed the following procedure: "Two dilutions, 1–2 and 1–4, are made of each serum. A single cell suspension of the corresponding cells is made by mixing one part of the whole defibrinated blood with an equal part of physiologic saline solution or by adding 3 parts of physiologic saline to one part of corpuscular sediment obtained by centrifugation.

Capillary pipettes are drawn out of Jena or Pyrex glass tubing (6 to 7

mm, outside diameter) the tips being of such a caliber that 100 drops of saline (pipette in vertical position) will measure about 2.5 cc. With such pipettes one drop of the 1–2 serum dilutions is mixed separately on an ordinary glass slide with one drop of the 1–4 blood cell suspension. Only two such tests should be made at one time on the same slide. An assistant is needed to note the time interval required for the first appearance of macroscopic clumping measured from the moment when the drop of serum and drop of cell suspension have been thoroughly mixed (with a knife blade) and spread over a circular area about 1.5 cm. in diameter. The clumping must be observed by looking down through the well-illuminated slide which should be held over a white surface. The mixtures are kept in motion by rocking the slide to and fro; cover glasses are not desirable.

If clumping occurs with the 1–2 serum dilution within one minute this result indicates that the respective serum is satisfactory for use as a grouping test serum. If under these conditions, clumping does not take place within one minute, the serum is discarded.

The method just described has been used in the Blood Donor Bureau of the Blood Transfusion Betterment Association, in New York City."

In testing B (anti-A) sera, it must be remembered that there are at least two grades of the A characteristic (sec. 1.2), and it is very important to have sera which react well with A_2. A number of fancied changes in blood group have probably resulted from false diagnosis of A_2 as O through the use of an unsuitable B serum. Therefore the sera should be tested if at all possible against a few A_2 bloods. If none are known, the B serum finally selected should be tested against 15 or 20 A bloods before actually being used.

2.222 Selection of Sensitive Blood Corpuscles

Blood corpuscles which react well with a strong test serum, may show themselves with a weak serum to be rather slightly sensitive. Therefore tests on weak sera should be done with blood corpuscles which are suitable for them. The test is carried out quantitatively, as in the choice of test sera. A certain agglutinating serum is chosen and the limiting titer determined with a number of different samples of the proper blood group. The blood corpuscles which are agglutinated by the highest serum dilutions are the most sensitive. The apparent absolute sensitivity may vary, but the order of sensitivity of different blood samples will, as a rule, be the same if the test is made with the serum of another person.

The absorptive power of corpuscles for the iso-agglutinins usually runs parallel to the agglutinability. Those blood corpuscles are the most sensitive which in the smallest amount can completely absorb a given amount of agglutinin. These differences may be important.

2.23 Preservation of Test Bloods

2.231 Serum

2.2311 Laboratory Preparations. The serum agglutinins outside of the body gradually decrease in activity. Warmth and light, as well as bacterial contamination, accelerate the deterioration. Properly preserved sera can, however, still be active after years under favorable conditions, although a definite time limit cannot be given. In general, of course, fresh sera are more desirable. A serum which has been preserved for a long time, and still reacts strongly with corpuscles of medium or high sensitivity, may at the same time be definitely weakened and fail to react with less sensitive corpuscles.

Sera keep without any particular precaution for a few days in a moderately cool room, or preferably in the ice box. They can be preserved frozen; where they are used routinely, however, it is more convenient to keep them in fluid condition at a temperature slightly above zero.

If it is desired to preserve sera for *weeks or months*, they may be taken with sterile precautions and well enclosed in good containers, sterile. The sera are put in small flasks in portions of a few cubic centimeters, or they are fused into ampoules of dark glass and kept in the ice box.

It is also possible to add chemical preservatives. It is then unnecessary to be so careful about the sterility. Sera may be preserved, and at the same time very clearly marked, by the use of dyes as recommended by Rosenthal (71) (cf. sec. 2.1). The procedure is described in sec. 2.3223. Sera have been preserved in an active state by this method for as long as five years. Preliminary tests indicate that sera possibly keep better preserved thus than when simply kept sterile in the icebox.

Sera which have been dried in the Flosdorf-Mudd apparatus also keep well. In some cases the state of preservation did not seem quite equal to that obtained with the dyes.

2.2312 Commercial Test Sera. Blood grouping sera can be obtained from various commercial houses. Some of them are very good. A few transfusion accidents have occurred as the result of the use of commercial sera which were too weak or incorrectly labeled, however, so it is very necessary that the investigator should personally convince himself of the activity of the sera, irrespective of statements on the package. It is always possible that deterioration has set in after the time of filling. Also the correctness of the label should be checked. Forssman found several incorrectly labeled sera from one firm.

The custom of filling capillary tubes with very small amounts, sufficient for only a few reactions, has the disadvantage that after the serum is used no control can be set up with that sample, should anything irregular

develop. One must be contented with testing other tubes from the same package, which is not entirely satisfactory.

2.232 Preservation of Blood Corpuscles

Blood corpuscles are best used fresh, only one or two days after being taken, but certain preservative steps may occasionally be helpful.

Corpuscles in general keep best in blood taken with sterile precautions and allowed to clot without the addition of anything. The clot is kept in the cold, and when corpuscles are needed, they are shaken out into saline. They may be kept thus about 4 to 6 days, and under the best conditions up to 2 to 3 weeks, but care should be exercised in using blood not entirely fresh. Corpuscles must be preserved somewhat above zero, because at freezing temperatures hemolysis sets in. For longer preservation, the blood immediately after being taken is fused into dry sterile capillaries. When corpuscles are needed, the clot is blown out into physiological saline.

Corpuscles from citrated blood may be kept, with sterile precautions, about 4 or 5 days or longer in the cold. 'Merthiolate' (Lilly) is useful as a preservative (1:5000). Longer preservation is possible with the solution of Rous and Turner: 3.8% sodium citrate solution 2 parts, 5.4% glucose solution 5 parts. To this is added 3 parts of sterile blood. If the blood is not sterile 'Merthiolate' may be added. Blood preserved in this way has been sent in satisfactory condition from North America to Germany. It should, of course, be kept cold.

2.24 Unknown Bloods for Tests

It makes some difference whether the blood may be taken when and as desired, or whether, as in certain medicolegal cases, one is dealing with blood stains or traces. We shall consider freshly taken blood here; the investigation of blood from corpses and blood stains will be treated later. The blood corpuscles are always tested and, whenever possible, the blood serum also.

2.241 Taking the Blood

2.2411 Venipuncture. The investigation is easiest when blood is available in amounts of at least a few cubic centimeters. Therefore, if the convenience of the investigator alone is consulted, the blood is taken by venipuncture, as is customary for the Widal and Wassermann tests. The blood may be put in sterile tubes and allowed to clot.

2.2412 From the Ear-Lobe. Some patients object to venipuncture; also, if many bloods are to be taken, it is time consuming. If it is wished to avoid venipuncture, a few drops of blood may be obtained by sterile puncture of the lobe of the ear.

The ear and the blood lancet (figure 6) are cleaned with alcohol or ace-

tone as usual. As soon as the solvent has evaporated, the dry ear lobe is pricked with the dry instrument. By slight pressure on the ear lobe, a few drops of blood are easily obtained, which are taken into dry glass tubes ("Widal tubes" or "Wassermann tubes") or into capillaries. Also glass capsules drawn to a capillary at each end, ("Wright capsules") are convenient. In this way it is possible to obtain 0.5 to 1 cc. of blood without difficulty. If it is desired to take the smallest possible amount of blood, or if it is not desired to test the serum, as in taking blood from infants, or from very anemic persons, one or two drops may be put in diluting fluid (see below) and a few "dry drop preparations" may be made on a microscope slide, to be tested later by the cover-glass method of Lattes (sec. 3.1185).

2.2413 From the Finger. Although there is probably less risk of infection, and less risk of a sensitive patient's fainting at the sight of blood, if blood is taken from the ear-lobe, some prefer to offer their fingers as a sacrifice on the altar of science. The blood is taken exactly as in sec. 2.2412, except that not so deep a puncture is needed. It is probably best not to use the fingers of laboratory workers. Patients in countries where elephantiasis is common may sometimes blame disease developing later on the blood taking. In such places the ear is to be preferred.

2.242 Obtaining Serum

The serum must be free from blood corpuscles before being tested. Clouding from other causes is not so important. It may be investigated either fresh or inactivated. The latter has the advantage that interference with the reading by hemolysis, which otherwise occasionally happens, is excluded.

Serum that has stood a few days, even without being inactivated, no longer acts hemolytically. According to Lattes (56) such serum has the advantage over inactivated serum that the "pseudo-agglutinins" have also disappeared. However, this is not important if the tests are made in the test tube.

2.243 Obtaining Blood Corpuscles

We recommend that the blood corpuscles never be used undiluted. Dilution protects against pseudo-agglutination and produces optimal conditions for true iso-agglutination, which does not develop so promptly and is not so strong with concentrated blood.

The blood is taken either directly into diluting fluid (1 drop of blood in 1 or 2 cc. of solution), or the corpuscles are shaken out of the blood clot into saline and added to the diluting fluid.

For diluting fluid, 0.9% salt solution is used, to each 100 cc. of which may be added 0.5 grams of sodium citrate. The sodium citrate is not

necessary, however, for if a few clots should form, they can easily be removed.

The *density* of the suspension may vary within rather wide limits. For the tests on the microscope slide, it is advantageous to have the concentration rather higher (say 3–5%) than for the reaction in the test tube. For the latter, a 1 to 2.5% suspension is satisfactory. An exact dilution could be made with the aid of a white-counting pipette; generally, however, exact measurement is not necessary. A few comparative tests may be made, and blood samples in the future diluted as estimated by the eye. A rule has been suggested: allow drops of about $\frac{1}{2}$ inch (about 1.3 centimeters) diameter to fall from a pipette onto a slide. Ordinary print should be legible through this. In doubtful cases, a weak suspension is safer than one that is too strong. Lund (61) found that a weak suspension giving a final concentration of 0.06 per cent was 8 times as sensitive as a 0.5 to 1 per cent suspension. Blood corpuscle suspensions should if possible be used on the day they are made, for in diluted blood bacteria grow very readily, and may cause nonspecific reactions (cf. sec. 2.2613).

2.25 Mixing Serum and Corpuscles for the Test

2.251 The Test Tube Method as the Method of Choice

2.2511 Differences in the Agglutinogens. In the isoagglutination reaction, we wish to allow the serum of one person and the blood corpuscles of a second person to react together, under the experimental conditions which are most suitable for the production and recognition of group-specific agglutination.

When there is no special reason to the contrary (sec. 3.118) the reaction is carried out in short test tubes (see sec. 2.1). Corpuscle-free serum is added to the amount of 0.1 cc. (2 drops) to a small test tube (see figure 13) and 0.2 cc. (4 drops) of the blood corpuscle suspension to be investigated is added. The mixture is well shaken and then centrifuged, preferably after the mixture has stood a few minutes. Centrifuging makes the reaction quicker and stronger, but is not indispensable for carrying out the reaction. If many bloods are being tested, as in anthropological work, it may be better simply to allow each rack of tubes to stand 15–30 minutes, then read (sec. 3.6513).

After centrifuging, the corpuscles are found as a sediment with a clear fluid above. To read, the sediment is carefully shaken up. It can then be seen whether clumps have been formed (positive agglutination reaction) or whether the corpuscles again disperse themselves evenly (negative agglutination reaction). Figure 10 shows a schematic drawing. It is practically always sufficient to make the readings with the naked eye. For greatest certainty that extremely weak reactions are not overlooked,

the (apparently) negative reactions may be reexamined under the microscope or with a hand lens.

In agglutination, the corpuscles are stuck together into a few rather flat aggregates, which with strong shaking float in the clear fluid. If they are *very* strongly shaken, the aggregates may break up into several smaller aggregates. Strong agglutination (+++) as shown in figure 10 is not at all uncommon, with the above technic, being rather the rule. Many sera give the same picture even when diluted 30 to 60 times.

Weaker agglutination (+ or ++) immediately after shaking, is also shown in figure 10. Even here, the reaction is so strong that no doubt

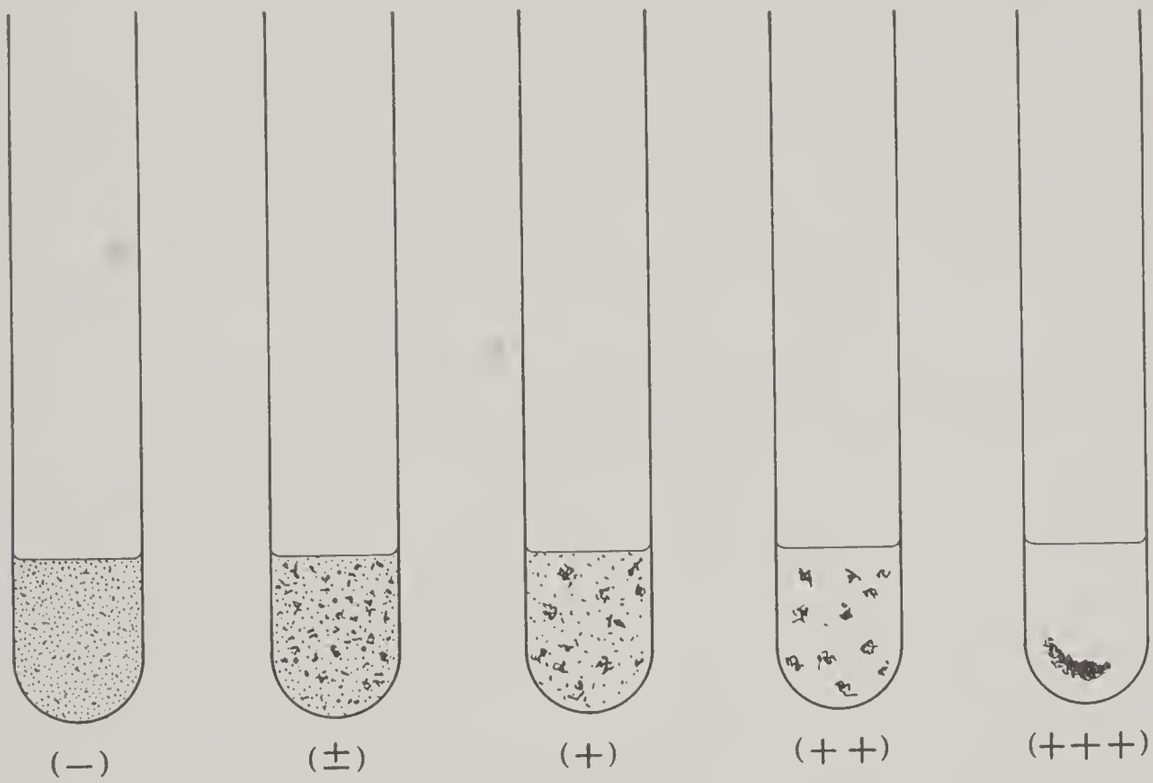

Fig. 10. Schematic representation of agglutination in the test tube

about the reading is possible. For comparison, along with the three positive tests there is shown on the left of figure 10 a *negative test*. Here the blood corpuscles on being shaken have evenly dispersed themselves; clumps are completely absent, and the fluid is evenly pink colored.

2.2512 Technical Details

2.25121 Centrifuging. The tests are centrifuged in the small centrifuge for at least two minutes, at 2000 r.p.m., or in larger machines at about 1200 r.p.m. Slower centrifuges could be used. In such cases it should be determined how long it is necessary to centrifuge to produce a definitely positive reaction with a weak serum. Centrifuging accelerates the progress of the reaction. The stronger reactions may be recognized without centrifuging, after a few minutes, but weaker reactions may still be absent or incomplete. By centrifuging, even with the weakly active sera, the posi-

tive and negative reactions are made recognizable almost immediately, without a long period of waiting.

2.25122 Test Tubes. We use small tubes of about 70–80 mm. length and 7–8 mm. internal diameter (sec. 2.1)

2.25123 Temperature. The reactions are carried out at room temperature. Only for special purposes are the tests done at 37° or 0°.

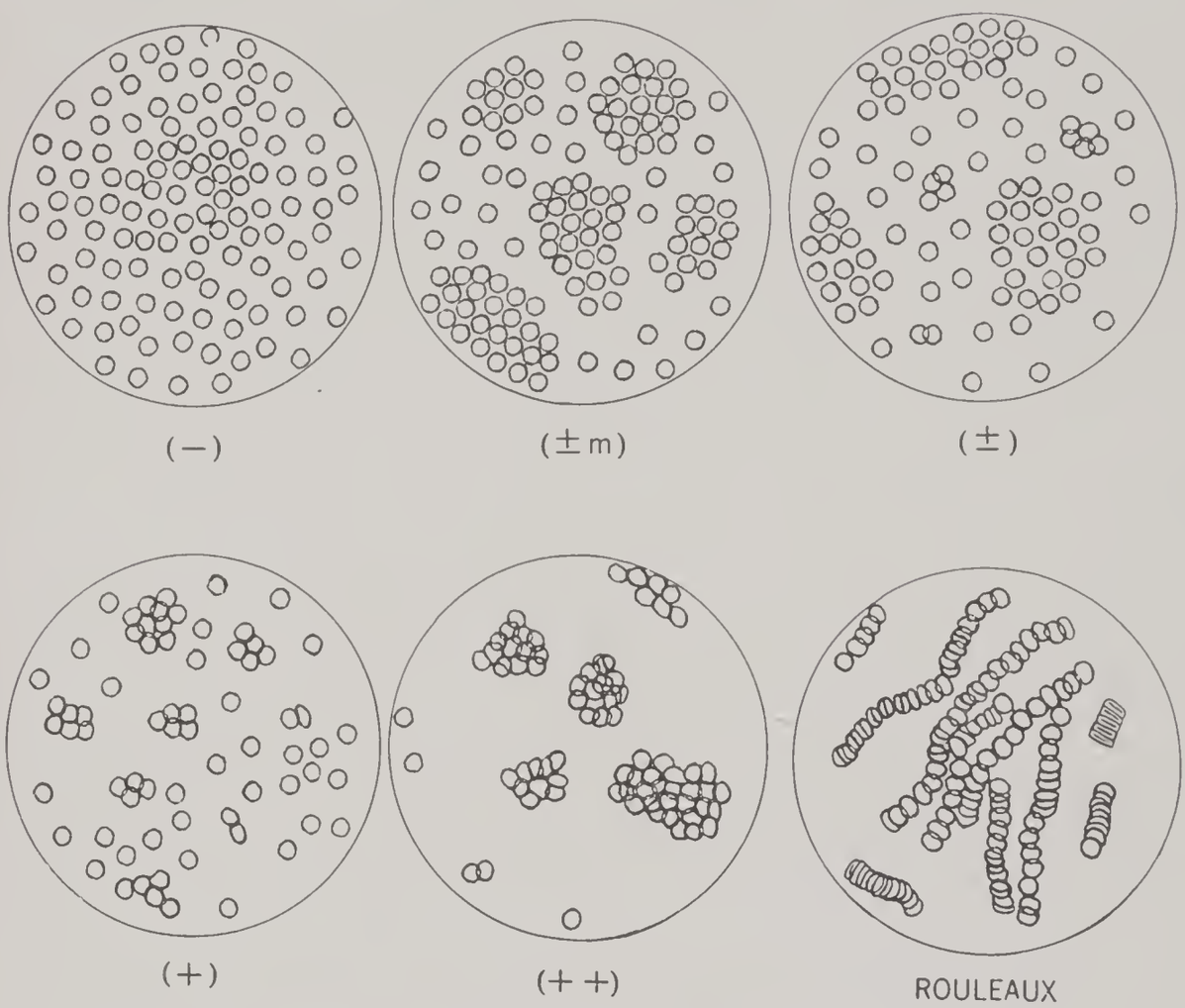

Fig. 11. Schematic representation of agglutination on slides

2.252 Slide Methods

Only in exceptional cases, for instance when the apparatus for the test tube method is not available, or the amount of material is limited (see sec. 3.1183, 3.1185), do we carry out the reactions on the slide.

2.2521 Moss-Lee-Vincent[1] Method. A drop of serum is placed on the slide, and a drop of corpuscle dilution added. By repeatedly tipping the slide, equal mixing is brought about. A positive reaction is often recognizable at once; it should appear at the latest after 5 to 15 minutes. If

[1] Moss worked with blood corpuscle suspension (1 drop of blood in 8 to 10 cc. of sodium chloride solution) in hanging drops. Lee and Vincent used undiluted blood on the bare microscope slide.

a longer period of observation is desired, the slide may be put in a moist chamber. Covering with a cover slip is not necessary, but is considered desirable by some (see 90). The reading may be done with the unaided eye (see fig. 22). Reactions which can be seen only with the microscope will not occur, except possibly in the case of the rare sub-groups, A_2, A_3, A_2B, A_3B, etc.

The microscope may be used to distinguish between agglutination and rouleaux formation (figure 1), although simple microscopic observation is not always sufficient; in such cases other special methods must be used (sec. 2.2611). The tests can also be carried out on "well-slides" (figure 9) (sec. 2.1).

2.2522 Rapid Test on Tile (70). Some Russian workers demonstrated to one of us in 1935 a technic for extremely rapid determination of blood groups, which has subsequently been described by Riddell (70). According to this author, the actual mixing of cells and serum is best made upon a white opal glass tile. This is smoother and more homogeneous than a porcelain tile, which owing to its granular surface may give rise to a deceptive appearance of agglutination under the hand lens. This is the most practical bedside method, according to Riddell.

A saucer or teacup turned upside down will serve in an emergency. In the USSR workers often used an ordinary white China plate.

A drop of serum is placed on the tile and whole blood is added to the serum. This is believed by its advocates to be a step towards greater accuracy and easier interpretation, provided that: (1) the typing serum is of known high titer, not less than 1–100, and (2) whole blood is used, instead of blood which has been diluted by drawing it off into saline or citrate.

The Medical Officer of the London Blood Transfusion Service has used this technic on approximately 10,000 donors, and has reported finding it consistently reliable.

Incubation is unnecessary provided that in cold weather the tile, etc., are warmed before use.

A time-limit is observed in reading the reaction. If the test is carried on for more than a few minutes, drying occurs at the edge of the patch and simulates agglutination, and the increased concentration in the center of the mixture may cause rouleaux formation.

2.2523 Lattes' Slide Method. (See below, sec. 3.1185.)

2.26 Sources of Error, Controls

Errors may be definitely excluded by employing not merely a single method but several methods simultaneously. It is sufficient in practically all pre-transfusion examinations to determine the groups and cross-match the bloods, where possible doing the former by testing both the cells and

the serum. In doubtful cases, it is desirable to carry out simultaneous tests with several different test bloods (sera and cells).

In the exceptional cases when a discrepancy is found, it should be recalled that rarely "defective types" occur where the agglutinins which would be expected according to the blood group scheme are absent. For example, we might find a blood of formula O (anti-B), or A (o). In such cases the blood group is determined by the *characteristics of the corpuscles* and not according to the agglutinins. In the report it is advisable to write explicitly the serum agglutinins found in the formula and to call attention to the anomaly by an exclamation point, thus: O (anti-A) !

A defective type should not be assumed after a single test. In most cases use of further reagents shows that the agglutinin or agglutinogen which was at first thought to be absent is present, although perhaps weakly developed.

A few additional points may be mentioned before we leave the subject of sources of error.

2.261 False Positive Readings

False positive readings are very rare with the test tube method, more frequent with the microscope slide method.

2.2611 Rouleaux. They are often due to the confusion of true agglutination with the so-called pseudo-agglutination due to rouleaux (sec. 1.11) formation (22). This characteristic piling up of the red blood corpuscles with their flat surfaces in contact is common in pregnancy and in many diseases, particularly infections. It depends principally on properties of the serum, as can be shown by cross tests. It also occurs intravitally, but as a harmless phenomenon. Rouleaux formation is essentially completely different from true agglutination; when found in cross-matching tests it does not show incompatibility. In rouleaux formation no absorbable agglutinin is involved (3, 63).

If the above makroscopic technic is employed, it is hardly possible to confuse rouleaux formation with true agglutination. But with the slide method, particularly if the serum and corpuscles are used undiluted, the mistake is commoner. As a rule the practised eye can distinguish the two under the microscope. But with the strongest degree of rouleaux formation the picture is almost exactly like that of true iso-agglutination. If the drop is diluted with a little saline, clumps due to rouleaux will separate into rouleaux, and greater dilution will cause these to break up also.

In addition to dilution, agitation, such as gentle stirring, will usually break up rouleaux, although they may re-form. The serum dilution required by the test tube method recommended here (1 part serum, 2 parts of cell suspension) automatically excludes this form of pseudoagglutination.

In tests made on the slide, a drop of saline may be added; or pseudo-

agglutination of this sort is abolished by lifting the cover slip and immediately replacing it. Usually a momentary slight pressure on the cover slip suffices; true agglutination is made stronger by the resulting motion, pseudoagglutination disappears.

A serum with a strong tendency to produce rouleaux or pseudoagglutination is not suitable as a test serum. This annoying property will disappear if the serum is kept at 37° for several hours (cf. 22).

2.2612 "Cold Agglutination". In addition to the above mentioned form of pseudoagglutination there may also be found a non-specific agglutination which depends on "antibodies", which may be absorbed in the cold ("cold agglutination" of Amzel and Hirszfeld, "panagglutination" of Mino). Since sera containing such agglutinins may agglutinate the cells of the same individual from whom they originate, (at ice box temperatures), these agglutinins have also been called "autoagglutinins".

TABLE 11

PROPERTIES OF DIFFERENT KINDS OF AGGLUTINATION (39)

Type of agglutination	Agglutinin left after absorption	Activity		Activity after moderate dilution
		Above 37°	Below 37°	
Pseudo-agglutination	+	>	<	−
Auto- and cold-agglutination	−	<	>	+
True iso-agglutination	−	≷	≷	+

> signifies increased, < decreased.

Compare also the remarks in sec. 2.512 on irregular agglutinins.

Cold agglutination is, under ordinary experimental conditions, rare at room temperature; it is certainly excluded at 37°, or if cold agglutination has already been produced at a lower temperature, markedly diminished. True agglutination is also weakened at 37° but not to the same extent.

Since cold agglutinins are usually weak, their action can also be avoided by working with a somewhat heavier cell suspension (5–10%). Some of the distinguishing properties of these various sorts of agglutination are shown in table 11, modified from Landsteiner (39).

Non-specific agglutination has also been observed with corpuscles which have been preserved too long. This pseudo-agglutination is due to bacterial contamination, and is important enough to describe in some detail.

2.2613 The Hübener-Thomsen Phenomenon (bacteriogenic agglutination). Hübener, Schiff and Halberstaedter, and Thomsen (33, 80, 82) observed that certain blood samples, especially dilute suspensions of corpuscles in saline, which had been kept for some time, became pan-agglu-

tinable; that is, the erythrocytes were agglutinated by practically any human serum, even of group AB, or by animal serum. On the other hand, they were not agglutinated, or agglutinated only slightly, by saline.

Thomsen found that this property was transmissible. It can be carried along in repeated passage by the addition of some of the "altered" corpuscles to unaltered ones. Friedenreich (24) found that the phenomenon was caused by the growth of certain bacteria, which he designated as J and M bacilli. Other organisms, such as certain vibrios, and a bacterium of the genus Corynebacterium, also had this effect (18).

The reaction is due to the "unmasking" of a new agglutinogen (T) in human (and animal) cells by an enzyme produced by the bacteria. Since practically all normal human sera contain the agglutinin anti-T, although it ordinarily never manifests itself, such altered cells are agglutinated, even by AB serum.

It has been shown (36, 18) that the anti-T agglutinin can be removed from sera by absorption with pan-agglutinable cells.

Davidsohn and Toharsky also found that an agglutinin, anti-H, for a normal receptor, H, present in all human (and some animal) erythrocytes, was produced by their Corynebacterium. A similar agglutinin was previously reported by Grove and Crum (28). The term "bacteriogenic agglutination" was proposed by Davidsohn and Toharsky (18).

If such altered corpuscles are tested in the usual way, with human test sera anti-A and anti-B, the group may be falsely reported as AB. Also, since certain sera may agglutinate the altered erythrocytes only weakly, a false diagnosis of A or B is possible.

The development of the bacteria and the resultant alteration of the corpuscles appears most quickly in erythrocytes preserved as a suspension in saline at room temperature. Sometimes a light lilac tinge denotes the presence of bacteria in the suspension. The alteration will usually not appear before 3 to 4 days at the soonest in undiluted blood.

Erroneous determinations due to bacterial alteration of the corpuscles will be avoided if the blood is tested while fresh. If the blood can not be tested while absolutely fresh, then it should be taken sterilely and the tests should preferably be done with sterile apparatus (glassware, pipettes, saline). Merely keeping the blood on ice after taking may not be sufficient.

A check on suspected pseudo-agglutination due to bacterial contamination consists in testing the cells with human AB serum, preferably with several such sera. If the cells being tested are all right for use, they will not be agglutinated by AB serum.

2.2614 A special type of non-specific agglutination may cause trouble in tests on cord blood. Cord serum is usually of a slightly gelatinous consistency due to admixture of substances from the cord. According to

Thomsen the characteristic of this non-specific reaction is that the "agglutination" appears when the slide preparation is brought into motion by stirring. The reaction disappears or becomes much weaker when the drop comes to rest again. In the test tube the reaction is much weaker. The non-specific character of the reaction is recognized by using group O cells as a control (Thomsen).

2.262 False Negative Readings. Cell Suspensions too Heavy. (See sec. 2.243).

An agglutinin which is adequately strong may fail, if it has to react with an excess of corpuscles. Therefore the cells should always be used in dilute suspension.

2.2621 Agglutinin-agglutinogen System too Weak. If one of the two components, the agglutinin or the agglutinable substance, or both, is too weak, errors are possible which can be of considerable importance, as in blood transfusions and questions of paternity. A positive reaction may escape notice if the iso-agglutinins are very weak, or the blood corpuscles very insensitive. Figure 5 illustrates the considerable individual differences which occur in the strength of the serum. Fortunately, however, the more extreme variations are rare in the adult. In some cases of leucaemia the agglutinin titer may be very low (81); in some cases the weak development of agglutinins is familial, and independent of disease.

Of more practical importance is the weak development of the agglutinins in early childhood. The newborn apparently never have any agglutinins of their own, though some of the mother's agglutinins may be present. The latter disappear in the first days or weeks of life, and the child's own agglutinins then gradually develop, but at first are very weak. Figure 12 shows the average strength of the agglutinins for different ages. The curves show that corresponding to the increase of titer in youth there is a decline in old age (senile involution—Schiff and Mendlowicz, 81). However the agglutinins of even very old persons are only rarely so weak as to cause diagnostic difficulties (87).

Even with a single individual some variation in the sensitivity of the blood corpuscles must be reckoned with.

There are persons with poorly agglutinable erythrocytes. In particular, false negative reactions may be obtained with a poorly developed A characteristic. This occurs, in a hereditary manner, in the subgroups of A and AB (cf. sec. 1.2 and sec. 2.5). About 20% of the individuals in group A and about 50% of group AB belong to this weak type, and A_2 in group AB is even more weakly developed than in group A.

Very rarely persons are found with an A definitely weaker than the average A_2 (the A_3 of Friedenreich; see sec. 1.22 and sec. 2.525). Here particular care is necessary. The attention of the investigator will usually

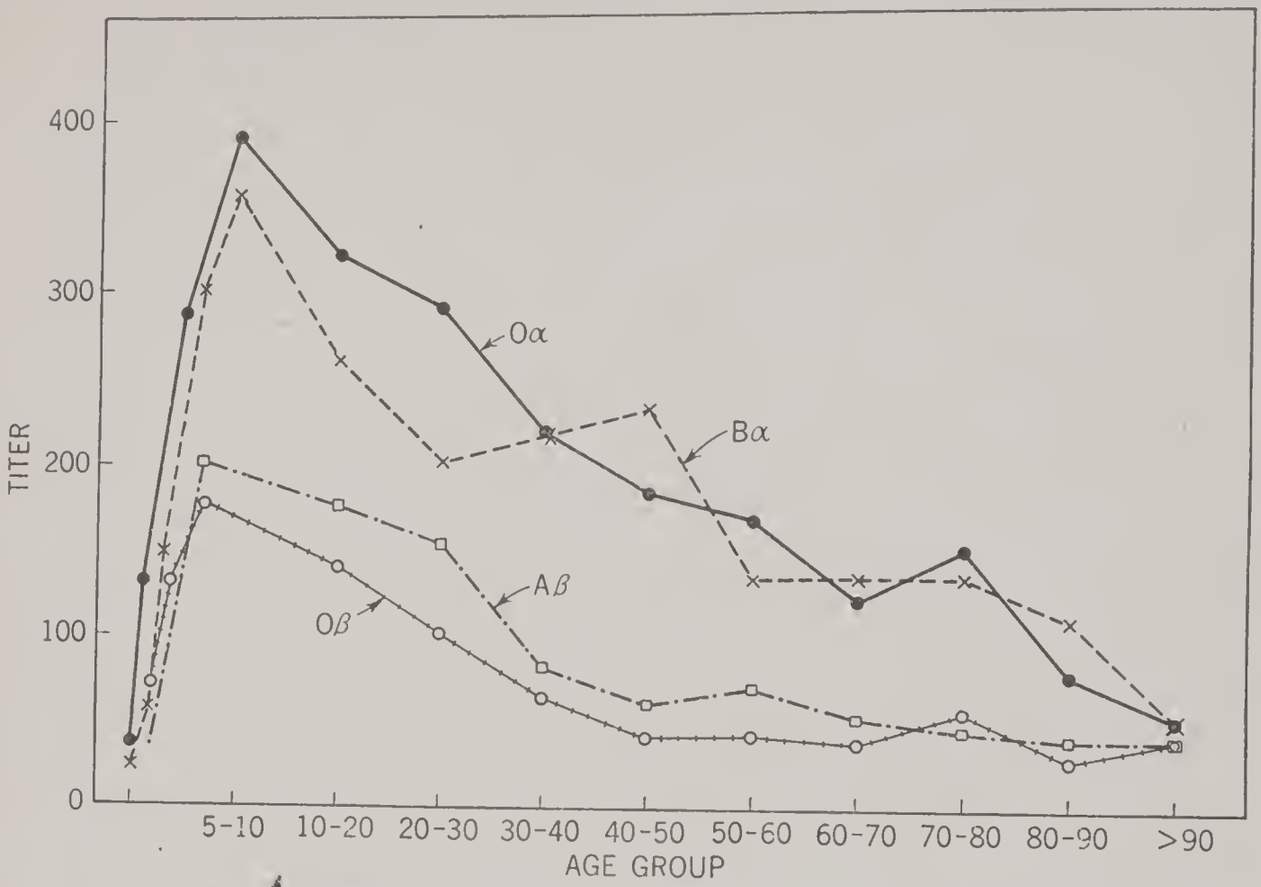

Fig.12. Strengths of agglutinins in individuals of various ages (after Kemp) (87)

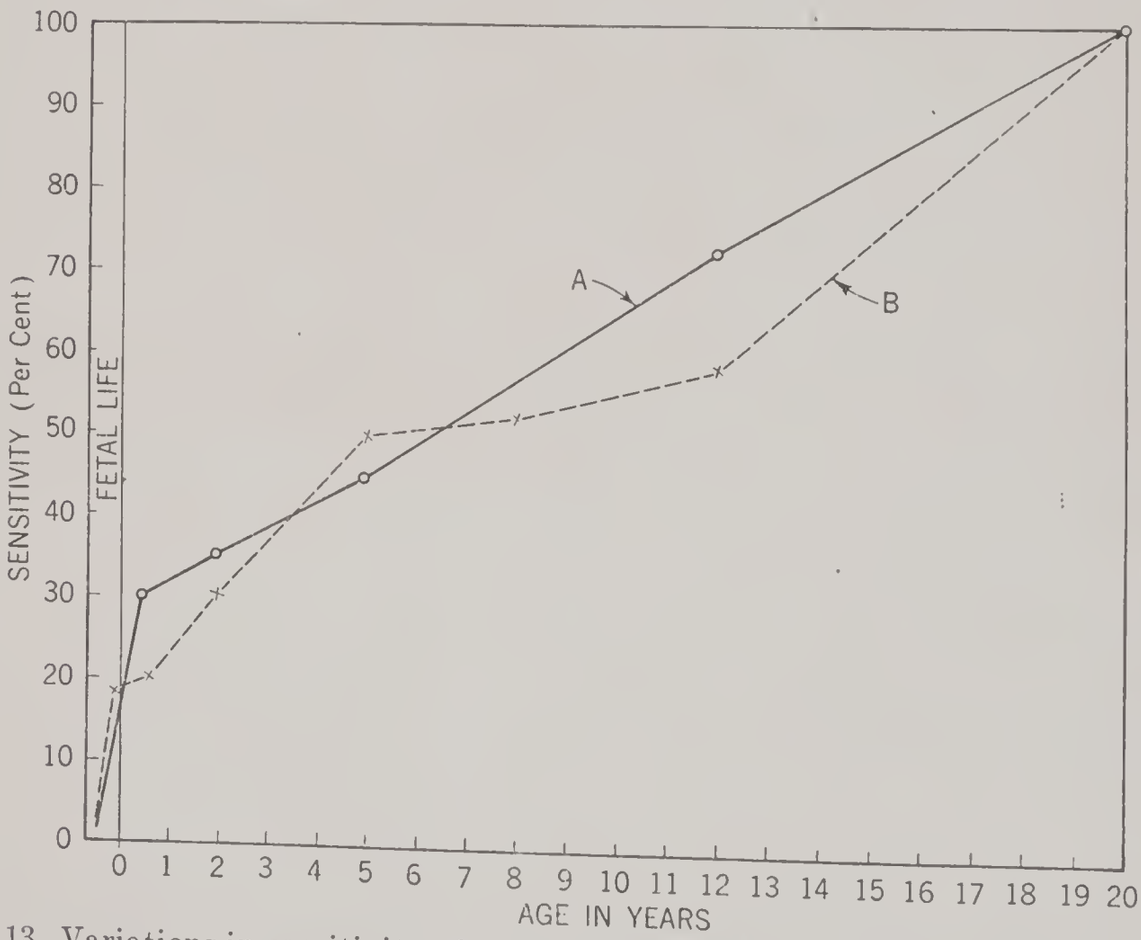

Fig. 13. Variations in sensitivity of corpuscles with age (after Thomsen and Kettel)

be attracted first of all by the discrepancy in his findings for the erythrocytes and the serum.

The erythrocytes of the newborn are somewhat less sensitive than those of adults. If the average sensitivity of the erythrocytes of adults is set equal to 100, then the lower sensitivity of the cells of the newborn and their gradual increase in sensitivity are shown in figure 13. In old age the sensitivity of the erythrocytes does not markedly decrease. In some newborn children, particularly in groups A_2 and A_2B, the sensitivity is so low that an A reaction is obtained only on test with the strongest sera, as for example with absorbed immune sera.

In all blood grouping work, the investigator should keep in mind from the beginning the possibility that either the serum or the corpuscles may be too weak, and strive to avoid error from this source, on the one hand by using strong, tested sera, and on the other by using fresh, highly sensitive erythrocyte suspensions.

In case of doubt, reactions obtained with human sera may be checked with immune sera. For standard erythrocytes of group A, sub-group A_1 should be used. The centrifuge method helps prevent error, by hastening reactions which might otherwise take several hours, or even escape observation. The reaction is somewhat more sensitive if the mixtures of corpuscles and serum are not centrifuged immediately, but first allowed to stand an hour or two at room temperature.

In special cases the findings made on the erythrocytes can be amplified by the investigation of other materials. In cord blood the erythrocytes generally react only weakly, but the antigen dissolved in the serum reacts as strongly as in adults (O. Thomsen). This can be studied with the help of the agglutinin inhibition method (sec. 2.75). Or anti-A precipitating sera (sec. 2.77) may be useful.

2.26211 Confirmation by Saliva Test. Investigation of the saliva may sometimes enable a decision to be made, if the cells are found to contain a definite B, but it is doubtful whether or not a weak A is also present, (differential diagnosis between B, anti-A, and AB, o). Diagnosis from the blood alone may be particularly difficult if there is also present a weak anti-A agglutinin (formula A_2B, anti-A_1). The saliva is then tested by the inhibition technic (sec. 2.75) for the presence of B substance. If B is present the individual is a secretor, and we may assume with certainty that the saliva will also exhibit A inhibitory activity, if the individual belongs to group AB. The A content of the saliva, even in subgroups A_2B or A_3, is strong enough so that its detection is always possible. (cf. 92).

If the above individual has no B in his saliva, on the other hand, he belongs to the class of non-secretors (sec. 1.43), and no A in the saliva is to be expected, so this method can not be applied.

2.2622 Error not Due to Weak Agglutinin-agglutinogen System

2.26221 Hemolysis. Even with strong agglutinin and well developed corpuscle characteristics, a positive reaction may be overlooked, if the serum is rich in complement, so that hemolysis takes place. Such a "paradoxical" negative reaction is obtained only with fresh serum; practically, therefore, it arises only in groupings preparatory to urgent transfusions. The investigator who is accustomed to looking only for agglutination might overlook the hemolysis, which is detectable by a clearing and reddening of the fluid. The pre-hemolytic stage, during which agglutination is repressed and the hemolysis not yet evident, is particularly critical.

This source of error can be excluded either by hastening the hemolysis by immersing the tubes in water at 37°, whereupon the hemolysis sets in immediately or after a few minutes,—at the latest after an hour; or by first inactivating the serum by heating it to 50–55° for at least 5 minutes; or by simple dilution of the serum.

A fivefold dilution of the serum is adequate to exclude disturbing complement action, and allow pure agglutination to appear. In testing very fresh sera it is therefore necessary to set up two tests, one with undiluted, and one with fivefold diluted serum. If no agglutinins are present, both tests will be negative; if agglutination has been inhibited by hemolysis in the undiluted serum, the test with diluted serum will be positive. If the agglutinins present are weak, then neither hemolysis nor pre-hemolysis, with attendant paradoxical negative reaction, will occur, but at least the undiluted serum will agglutinate (O. Thomsen).

Weak reactions are most certainly avoided by selecting for the test bloods the most highly active sera and/or particularly sensitive corpuscles. This can be done most accurately by titration of a series of sera and corpuscles.

2.263 Controls

False agglutination can be certainly recognized, if two kinds of controls are used.

1. Controls of the blood corpuscles by mixing them with their own serum and with serum of group AB(o). If the group of the corpuscles being used is known, any sera of the same group may be used for the control.

2. Controls of the serum by testing it with inagglutinable corpuscles; that is, with those of its own group or of group O.

If pseudoagglutination is present, tests 1 or 2, or both, will be positive.

Since there are individual differences in the strength of pseudo-agglutinations, multiple controls, including the bloods of several different persons, may be necessary in some cases.

In some circumstances there should be added:

3. Control for cold agglutination in its different forms, by setting up a parallel experiment at 37°. The test serum, if less than two weeks old, must be inactivated; centrifuging is omitted. Observation period, 2 hours.

2.264 Deviations from the Group Scheme

Thus we may designate all those rare cases which really or apparently do not conform to the Landsteiner rule. We may distinguish two principal types of deviation.

2.2641 Defective Types. First, there is the so-called defective type. Here some one of the blood group characteristics which would be expected according to the Landsteiner rule is absent; for example, blood formulas O (anti-A), or A(o). Such a formula is not unusual in the newborn and in young children but in older persons it is extremely rare. Such an anomaly may be falsely assumed in cases where an agglutinin is weakly developed.

2.2642 Extra Factors. A second type of deviation consists in the presence of an agglutinin or agglutinogen not consistent with the Landsteiner rule. Here belong, for example, such bloods as A_2B (anti-A_1). Such atypical groups result from the presence of "irregular" agglutinins. These act on many, but not all, individuals of a certain group; this distinguishes them from typical group agglutinins. The irregular agglutinins often possess a recognizable specificity, most usually for the characteristic A_1, more rarely for A_2. In the latter case they usually react also with O. Occasionally an "extra agglutinin" for the factor P, described by Landsteiner and Levine (42) is seen, and extremely rarely an agglutinin anti-M. So we may find such bloods as A_1B (anti-A_2) and A_2B (anti-A_1). Most of these reactions can be classified as "cold agglutinations"; they disappear at 37°. Atypical reactions of this sort may be avoided if only test sera known to contain no irregular agglutinins are used.

The irregular agglutinins are usually definitely weaker in strength of agglutination and in titer than the typical group agglutinins. With the technic recommended here, involving centrifuging followed by shaking, the weakest reactions, and thus in general the atypical ones, tend to disappear, so that the irregular reaction usually escapes notice. The very rare strong irregular reactions are, however, recognizable after centrifuging.

If it is wished to obtain the irregular reactions systematically, the tests are set up in test tubes, but not centrifuged; the tubes are kept in a water bath at 20° and occasionally shaken, and finally a drop is examined microscopically. The drop may be taken out and placed on a slide with a glass rod (40, 44).

The frequency with which cold agglutinins can be found depends on the

sensitivity of the technic employed, and the number of different erythrocyte suspensions each serum is tested against.

Kettel was able, by his technic, to find such agglutinins in most sera; in a small test tube was placed 0.1 cc. serum and 0.1 cc. of a freshly made very thin ($\frac{1}{2}\%$) suspension of erythrocytes. Mixtures were kept, not over 12 hours, at 0° and 13°.

The so-called deviations from the group scheme have thus far had no practical application. The reactions involving them are obviously basically different from typical isoagglutination, and if the ordinary technic is used, will not be confused with them.

In persons who have previously received blood transfusions it is possible that immune agglutinins of special specificity will be found. Such cases have been described by a number of workers, including (46, 65, 60, 93). These agglutinins may differ from the cold agglutinins described above; they were active in some cases at 37°, in others more active in the cold. They seem not to be permanent, but disappear from the serum after a time. Such irregular immune agglutinins are rare, at least in any strength, but they are of some practical importance, because a patient with such antibodies might be harmed by a transfusion from a donor of the same group. Unexpected agglutination found on cross-matching patient and donor has in some cases been traced to anti-Rh agglutinins (sec. 3.115).

The antigenic factors *independent of the scheme of the classical four groups* which may be detected with the aid of suitable immune sera, and which make it possible to separate blood into other groups (the M and N factors of Landsteiner and Levine) are described in sec. 2.4 in more detail. These factors are not a source of error in ordinary group determinations, because normal antibodies for them are not present*. (cf., however, 91).

2.3 Determination of Blood Groups with Immune Sera

For blood group determinations on fresh blood, normal human sera are generally used. It is however possible, as in other serological procedures, to use group specific sera obtained from animals.

Such sera, produced at first for theoretical studies, have been found useful in anthropological and medicolegal investigations, and they are recommended, at least to confirm doubtful tests, for the routine groupings preliminary to transfusion. The blood types M, MN, and N are regularly determined with such immune sera, since human isoagglutinins for M and N are lacking, and even for testing for A and B, sera can be obtained which are definitely stronger than strong normal human sera. Such sera are especially useful when it is wished to detect weakly developed group

* Exceptions to this rule (a normal anti-M agglutinin) have been recorded in only three instances.

characteristics, as for example the factor A_2, particularly in persons of group AB and in young children. Since these immune sera are used in diluted form, they are economical to use, and when properly prepared do not give either pseudoagglutination or non-specific reactions, which sometimes are a source of difficulty with normal serum.

The immune anti-A and anti-B agglutinins produced by injecting A and B erythrocytes into rabbits are not identical in all respects with the anti-A and anti-B in human sera. The human B antigen, for instance, has been separated into 3 hypothetical fractions (sec. 3.513), which we may designate as B_i, B_{ii}, and B_{iii}. Friedenreich states that B_{ii} and B_{iii} occur in the blood of the rabbit (and probably of other mammals). Consequently an anti-B serum from the rabbit will be anti-B_i (*horror autotoxicus*). But since the fraction B_i is so far as we know found in all human bloods of group B, this does not matter, if the sera are to be used only for reactions with human corpuscles. We have compared anti-B sera from rabbits with human A serum in hundreds of cases without finding a single discrepancy. It is further claimed that both fraction B_i and B_{ii} are lacking from chicken blood, and that injection of rabbit cells into chickens will consequently produce an anti-B_{ii} serum.

Human A (and O) sera usually contain anti-B_{ii} and anti-B_{iii}, not always anti-B_i.

We may use immune sera with every confidence in testing human erythrocytes; in other work, such as inhibition tests and investigations on anthropoids, a thorough knowledge of the behavior of each serum is requisite.

2.31 Kinds of Immune Sera

Immune sera may contain in addition to agglutinins other immune antibodies which can be applied for special purposes in group determinations. We may mention particularly: hemolysins, complement-fixing antibodies, and precipitins.

We may divide immune sera into *isogenetic* and *heterogenetic* sera. An isogenetic immune serum is employed to test for the same antigen which was injected into the animal in preparing it. For example, we may inject a rabbit with human blood corpuscles of group A and obtain specific agglutinins directed against human blood corpuscles of group A and group AB. Such sera contain, in addition to the group specific antibodies, species specific antibodies for human blood. These can be removed by selective absorption with human corpuscles not containing A (e.g. group O). Then a pure group specific agglutinin remains.

Selective absorption is not usually required when a *heterogenetic* serum is used. An antibody is called heterogenetic when it reacts with another antigen than the one which was used for immunization. The classical

example is the Forssman sheep hemolysin, which is obtained by injecting rabbits with an extract of horse kidney.

In blood group determinations a heterogenetic serum can be used for the detection of blood group characteristic A. Group specific immune agglutinins for A are obtained by immunizing suitable rabbits with sheep cells. Such sera are group specific from the beginning for human blood of group A, and do not require absorption. Also, by injection of rabbits with human cells of group A, antibodies (hemolysins) for sheep blood are obtained. Such sheep hemolysins are specifically absorbed by erythrocytes of group A. For the use of these "A-specific sheep hemolysins", see sec. 2.754.

2.32 Production of Group Specific Immune Sera

It is best to choose rabbits which are not too young, and to immunize about 5 to 10 rabbits, since not every animal will produce group specific antibodies. The capacity for the formation of strong antibodies for group A seems to be hereditary (88). If a breed of rabbits selected for this capacity is not available, then those rabbits are selected which already possess antibodies for A. Their serum should also be free from inhibiting effect on the agglutination of human red cells of group A by human serum of group B. Another point worth considering is, does their serum contain sheep hemolysins? Animals which fulfill all three of these conditions almost always produce good A-specific sera.

Satisfactory sera for absorption are successfully produced, in rabbits selected at random, in somewhat less than half of those injected with A blood. If the preliminary tests mentioned above are used as a basis of selection, the proportion of successes is much greater. Good anti-B sera are somewhat harder to produce.

For the production of anti-B agglutinin rabbits should be chosen which already possess agglutinins for human B cells. According to Battey, Stuart and Wheeler (2), all such animals will form group specific anti-B agglutinins. Of the animals which do not possess anti-B in advance, only about two-thirds or less will produce group specific anti-B.

It is best to test a large number of animals at one time. Out of 15 or 20 animals as a rule at least 1 or 2 will be found suitable.

2.321 Preliminary Tests of the Animal's Sera

2.3211 Test for Pre-formed Agglutinins. Test the serum of new rabbits for agglutinins for human erythrocytes. The serum is inactivated (20 minutes at 56°), diluted, and used in 0.1 cc. amounts. A 2% erythrocyte suspension is used in 0.1 cc. amounts. The tubes are allowed to stand 10 minutes at room temperature and then centrifuged.

Example: Test of sera from 6 rabbits with human cells of groups O and A.

Tested with human red cells, group O

Serum, diluted	Rabbit serum number 1	2	3	4	5	
1:2	—	—	—	—	—	—
1:4	—	—	—	—	—	—
1:8	—	—	—	—	—	—

Tested with human red cells, group A

	Rabbit serum number 1	2	3	4	5	6
1:2	—	+++	++	—	+	—
1:4	—	++	+	—	±	—
1:8	—	++	—	—	—	—

Sera 2 and 3 contain anti-A agglutinins, indicating that these rabbits are fitted for immunization. A rabbit giving a weak reaction, as number 5, should not be used.

2.3212 Test of Normal Rabbit Sera for Sheep Cell Hemolysins. The sera are inactivated (water bath at 56°). Dilutions 1:5 and 1:50 are prepared, and decreasing amounts (0.5 to 0.1 cc.) placed in test tubes. Saline is added to make the volume 0.5 cc. To each tube are added sheep cells (0.25 cc. of a 5% suspension of washed cells). As complement, fresh guinea pig serum diluted 1:10 is used (0.25 cc.). After shaking, the tubes are placed in the incubator for 20 minutes.

(a) Typical set-up for one serum

Serum dilution	Test tube number 1	2	3	4	5	6	7	8	9 (control)
1:5	0.5	0.25	0.15	0.1	—	—	—	—	—
1:50	—	—	—	—	0.5	0.25	0.15	0.1	—
Saline	—	0.25	0.35	0.4	—	0.25	0.35	0.4	0.5
Sheep cells 5%	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Guinea pig serum 1:10	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25

(b) Testing of 6 rabbit sera for normal sheep hemolysins

Serum dilution	Amount	Rabbit serum number 1	2	3	4	5	6
1:5	0.5	c	c	0	str	c	w
	0.25	c	c	0	w	0	0
	0.15	str	c	0	0	0	0
	0.1	w	c	0	0	0	0
1:50	0.5	0	str	0	0	0	0
	0.25	0	a.0	0	0	0	0
	0.15	0	0	0	0	0	0
	0.1	0	0	0	0	0	0

Readings after 60 minutes incubation at 37°.

0 = no hemolysis; a.0 = almost no hemolysis; w = weak hemolysis; str = strong; c = complete hemolysis.

2.3213 Test for Group Substances. Explanation of the test of rabbit sera for the presence of substances inhibiting the A-hemolysis by group specific human serum follows: The rabbit sera were first kept in a water bath at 60° for an hour, then diluted 1:5. Decreasing amounts of this dilution, as indicated below, were examined for their inhibiting effect on the A-specific hemolysis of sheep cells (cf. sec. 2.31).

(a) Set-up for one serum; five tubes are required

	Test tube number				
	1	2	3	4	5
Heated rabbit serum, dil. 1:5	0.5	0.25	0.15	0.1	—
Saline	—	0.25	0.35	0.4	0.5
Diluted A-specific immune serum	0.5	0.5	0.5	0.5	0.5
Sheep cells 5%	0.25	0.25	0.25	0.25	0.25
Fresh guinea pig serum 1:10	0.25	0.25	0.25	0.25	0.25

The serum is diluted as described in sec. 2.7541, so that hemolysis occurs within 20 minutes. The mixtures are incubated for 20 minutes at 37°.

(b) Test of 6 normal rabbit sera for presence of substances inhibiting the A-specific hemolysis of sheep cells

		Serum number					
	Amount	1	2	3	4	5	-
Dilution of heated rabbit serum 1:5	0.5	0	c	c	0	0	0
	0.25	0	c	c	0	a.0	0
	0.15	0	c	c	a.0	w	0
	0.1	0	c	c	str.	c	0

0 = no hemolysis; a.0 = almost no hemolysis; w = weak hemolysis; c = complete hemolysis.

Sera number 1, 4, 5, and 6 inhibit the A-specific hemolysis, and therefore contain group A substance. These rabbits should not be used for immunization with A cells, or with sheep cells.

2.322 Technic for Injection and Bleeding of Animals

2.3221 Injection. Rabbits are almost always used for this work, and probably possess definite advantages over other animals, such as guinea pigs, which are too small, and furnish too little blood, for common use. Some other animals do not seem to produce satisfactory immune hemagglutinins (90).

It is probably best to inject the antigen intravenously, using other methods only as supplementary to this.

Before starting the injection a syringe (a 2 cc. or 5 cc. type is suitable) and needles (probably 25 gauge is best, though there are those who prefer gauge 20) are boiled 20 minutes to sterilize them. Modern syringes may be boiled assembled.

The injection is made into the marginal vein of the rabbit's ear. During this procedure many rabbits will sit quietly in the operator's lap, others may require a "rabbit box" (sec. 2.1) or rolling in a towel. If the vein is hard to see or if difficulty is experienced in inserting the needle into the vein, a *little* xylene may be rubbed on the ear with cotton, then washed off with alcohol. (In any case a little alcohol, by laying the fur, makes the vein more visible.) If the vein does not come up, a little rubbing with dry cotton will now bring it up. (The xylene and rubbing,—*v. infra*—, essential for bleeding, are not usually necessary for injecting.) The needle is inserted into the vein, in the same direction as the circulation (towards the heart), the ear and needle held with the left hand so that the thumb is over the point of entry, and the antigen injected, slowly at first. Resistance indicates that the needle is not in the vein. Any injection into the tissues around the vein is likely to destroy the future usefulness of the vein for injection.

The same ear (right or left) is always used for injection, the other being reserved for bleeding.

In some cases intraäbdominal injections are desirable. The rabbit is held, belly outward, between the operator's knees, and the head and forefeet held with the left hand. Enough alcohol to wet a small area of fur is dropped on the belly, the (20 gauge) needle of the already charged syringe is inserted, first through the skin, then through the abdominal wall, and the injection made with the right hand.

2.3222 Immunization. We use the following routine: rabbits, selected as described above, are injected thrice weekly with a 30 per cent suspension of thrice washed, packed erythrocytes of proper group. After the first week, the first injection of each week is given intraäbdominally (3 cc.); all other injections are intravenous (1 cc.). One week after the twelfth injection the rabbits are bled from the ear vein (50–80 cc. of blood), and the serum tested (see below). If it is good, the rabbits are bled also on each of the following two days. If the serum is weak, the rabbits may be injected another week or two; if no specific agglutinins are found in an animal, however, it is best to discard the animal, and substitute new rabbits, if necessary.

2.3223 Bleeding. Blood may be taken from the rabbit's heart, with a 50 cc. syringe and large (gauge 18) needle, but occasionally an animal will die. Almost as much blood may be taken, though not so quickly,

from the marginal vein of the ear, if the technic described under *injection* is used to engorge the vein. A longitudinal (about 2 to 4 mm.) slit is made in the vein with a sharp scalpel or the blade of a Bard-Parker knife. If a clot forms in the incision it should be scraped off. If the flow stops, another treatment with a little xylene, and friction, may revive it. In this way 50 to 80 cc. of blood may be taken. Similar bleeding without a rest period (and preferably another injection) may result in the death of the animal. If it is wished to bleed the animal to death, most blood is obtained by inserting a canula in the carotid or femoral artery.

2.3224 Preservation of Antiserum. If blood is taken from the heart with a sterile needle and syringe, it will be sterile, otherwise it may not. If it is not, the serum should be treated to preserve it, preferably by passage through a Berkefeld filter, followed by storage in a sterile container in the icebox. Addition of 'Merthiolate' (1:5,000) seems to be satisfactory. (Phenol is likely to cause clouding.)

It is apparently the impression of many that anti-M and -N sera, after absorption, can be kept only a few weeks. Since the production of such sera is somewhat difficult, and involves the use of a considerable amount of human blood of definite type, a method of preservation is important. We have already referred (sec. 2.231) to Rosenthal's (71) proposal for the simultaneous marking and preservation of human group A and B sera by the addition of antiseptic dyes. These dyes can be used successfully to preserve also anti-A and -B, and anti-M and -N sera.

Rosenthal recommended adding 0.01 cc. of a one per cent aqueous solution of neutral acriflavine to each cc. of A serum (anti-B), and 0.02 cc. of a one per cent aqueous solution of brilliant green to each cc. of B serum (anti-A). He also added basic fuchsin to the A serum, simply to intensify the red color. We have found it convenient to add to each cc. of A serum, or immune anti-B, 0.015 cc. of the acriflavine, and to each cc. of the B serum, or immune anti-A, 0.02 cc. of the brilliant green. To each cc. of anti-M or -N serum we added 0.015 cc. of the acriflavine. Absorbed antisera preserved in this fashion have been found in usable condition after as long as 5 years. Boyd considered that such sera kept better than those preserved with toluene, but Levine (57) reported that toluene was equally good. Sera have also been preserved satisfactorily by drying in the Flosdorf-Mudd apparatus.

Before use of preserved sera which have not been used for some time, they should be tested against suitable controls to guard against sudden losses of strength.

2.323 Tests of Immune Sera

The sera, if less than about 4 weeks old, are inactivated before testing (30 minutes at 56°).

2.3231 Agglutinins. Immune sera for A are tested with A cells, sera for B with B cells. To each 0.1 cc. of serum dilution is added 0.1 cc. of blood cell suspension. After 10 minutes at room temperature, the mixtures are centrifuged. The sera are strong enough if they agglutinate strongly in a dilution at least as high as 1:1000. Anti-sheep sera, which are to be used as anti-A agglutinins, may be used without further treatment. The smallest dose which gives strong agglutination (clear fluid remaining after shaking) is determined, and the serum used in 10 or 20 times that strength. For example, if it is found that the last dilution giving ++++ agglutination is 1:1000, then the working dilution will be 1:50.

The working dilution must be tested against a series of bloods of groups O and B. It must be negative against all these, or else a somewhat higher dilution must be used as the working dilution.

Sera from rabbits which have been immunized with human blood must be rendered specific by absorption. For anti-A sera may be used bloods OMN or BMN; for anti-B, bloods OMN or AMN. According to the strength of the agglutinins the sera are diluted 1:15 to 1:25 absorption. Complete absorption of 20 cc. of the 1:20 diluted serum will as a rule require 8 to 15 cc. of washed packed erythrocytes, best added in portions. Occasionally sera are found in which it seems best to do the last absorption at room temperature. A more detailed account of the preparation of absorbed immune agglutinating sera is given in the section devoted to M and N (sec. 2.4411).

2.3232 Hemolysins. We may confine our attention to sheep hemolysins produced by injecting rabbits with human blood of group A (7, 8). After the serum has been inactivated, it may be mixed with complement in the following amounts, and tested against sheep cells:

Dilution	Amount in cc.			
1:100	0.50	0.25	0.15	0.10
1:1000	0.50	0.25	0.15	0.10

The volume of each tube is brought to 0.50 cc. by addition of saline. In a control tube is placed 0.50 cc. of saline alone. Then to all tubes is added (1) 0.25 cc. of 5 per cent sheep cell suspension; (2) 0.25 cc. of fresh guinea pig serum 1:10 (complement). After thorough shaking, the tubes are incubated 20 minutes at 37°.

A good serum will produce complete hemolysis in the tube containing 0.15 cc. or less of the 1:1000 dilution.

There should be no trace of hemolysis in the control tube, which contains only saline, complement and cells, thus confirming the absence of hemolysins in the guinea pig serum.

When an immunized animal has produced a serum having approximately this titer, it may be bled out, and the serum passed through a Berkefeld and stored in the icebox. If the titer is too low, further immunization will usually raise it.

2.324 Other Types of Heterogenetic Sera

The chief objection to the preparation on a large scale of such immune sera is the large amount of human blood needed for the injections and absorptions. Especially in the case of anti-A and -B sera, the amounts required would be very considerable if it were attempted to use them for all routine groupings, though the proportion of blood needed, compared to that needed for obtaining human serum, would be only about as 1.5 to 1, and the resulting sera are usually stronger, and keep better. However, substances related serologically to the A and B of man occur also in various animals, and it is thus possible to produce useful heterogenetic sera without injecting human blood. It has been found (79) that anti-Forssman sera will agglutinate A and AB bloods specifically. Group specific antisera have been prepared by injecting horse and human saliva, and gastric juice (75).

Slight differences in these animal antigens will not matter, if all human bloods of the same type react in the same manner to the antibodies (cf. sec. 2.3).

2.3241 Agglutinins. Boyd (4) injected rabbits with filtered sterile saliva of types A and B from horses and from people (secreting type). To enhance the antigenic stimulus, an unrelated protein, *Limulus* hemocyanin, was mixed with the horse B saliva injected into some of the animals, and other rabbits were injected with a mixture of *Limulus* hemocyanin and a peptone (Witte) shown by the inhibition test to contain a relatively large amount of A substance.

The animals injected with the peptone-hemocyanin mixture, and one of those receiving the horse saliva of type A, produced a fair titer of group specific agglutinin, with very little non-specific agglutinin. It was found that the non-specific agglutinins could be removed by absorption with very small quantities of human erythrocytes, or their effect could be avoided by diluting the serum before use. Tests showed that such sera were entirely suitable for grouping unknown bloods, or for the indirect determination of the groups of dried blood and tissues.

The anti-B sera were not quite so good, for it is in general more difficult to obtain anti-B in rabbits. They exhibited definite group specific agglutinins of moderate strength however.

2.3242 Precipitins for the A Characteristic. Schiff (73, 78) discovered the existence of precipitins for the group substance A.

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If a strong immune serum is overlayered with a fluid which contains dissolved group specific substance, a disk-like precipitate, the so-called "ring" is formed. If the two fluids are mixed, instead of having one layered over the other, the mixture clouds, and later a precipitate is formed (see below, sec. 3.4131).

In this manner it can be demonstrated that human blood serum contains group specific antigen. Group-A-specific immune serum forms a ring if it is overlayered by the serum of a person of group A or AB who belongs to the secreting type. So far precipitating sera have been reported only

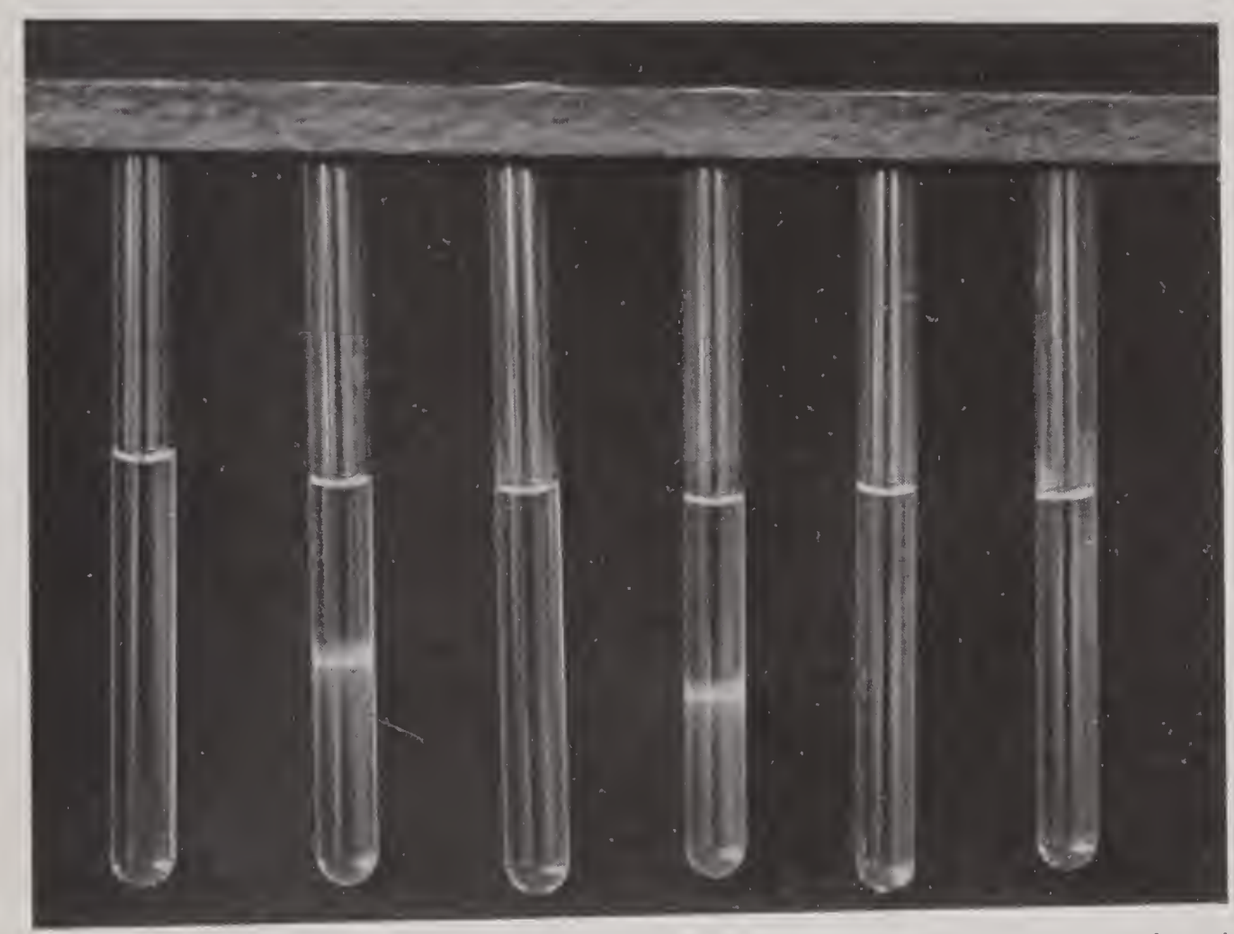

Fig. 14. Precipitin reaction with anti-A. Positive reactions in tubes 2 and 4 with A-containing saliva from secretors.

for A, but not for B. The immune sera are usually group specific from the beginning, or they can be made group specific by absorption with O cells (see figure 14). Boyd (4) examined 20 potent antisera made at various times for the preparation of group specific agglutinins (see above) and found precipitins for A substance in 7 out of the 10 anti-A sera examined. The strength of the reaction is generally greater with horse saliva than with human saliva, indicating that possibly the A substance is more concentrated (or more reactive) in the former.

These precipitating sera can be used occasionally to check the finding of A substance in body fluids, etc.

2.4 The M, N Types

2.41 General Considerations Relative to Factors Detectable by Immune Sera

In addition to the blood group characteristics A and B, other type specific blood corpuscle characteristics are known. Since normal human serum contains, as a rule, iso-antibodies only for A and B, other characteristics can not be detected merely by normal human blood or serum. Immune sera are needed. A prototype for such work is found in the experiments of Ehrlich and Morgenroth (21) who immunized goats with the blood corpuscles of other goats, and obtained *iso*-lysins, which acted only on the blood of certain goats. Obtaining immune iso-antibodies in man is not practical, so the new blood types are detected by sera prepared by immunizing animals with human blood, in other words with the aid of *hetero*-immune antibodies. If rabbits are immunized with the blood of a definite individual, species specific antibodies agglutinating all human blood are regularly obtained. In addition, however, in many cases type specific antibodies are produced. Either may be removed by selective absorption. By such means several type specific properties have been found. The most important corpuscle characteristics detected by these methods are designated by the letters M and N (41).

2.42 Locating Known M and N Bloods

If donors of known type are not available, extensive experiments are needed to find any new blood characteristic for the first time. Rabbits must be immunized with the blood of certain persons and the resulting sera absorbed. It is attempted to find bloods which will absorb all antibodies against themselves, but will leave antibodies in the serum which are active against the blood of other persons. If such bloods are found, then type specific antibodies are present in the serum which may now be identified by comparison with blood of known types (A, B, M, N, and other characteristics to be mentioned later).

It is generally more practical for an investigator to have samples of anti-M and -N serum sent to him from the laboratory of some qualified worker. If possible those intending to begin experiments with M and N should have the technic demonstrated to them by some one familiar with it. A number of bloods from persons who will later be available for testing may be typed at the same time.

2.43 Stability, Division of Bloods into Classes, Heredity

The characteristics M and N are already developed in the newborn. They are not changed during the course of life; disease is without effect on them.

M and N do not occur entirely independently of each other, as shown by the fact that at least one of the factors is always present. We may distinguish three classes:

M, N, and MN

The most frequent class is the MN, which in European stocks runs about 50%. Class M is about 30% and N about 20%. The characteristic M is much more frequent in the American Indian, and N less frequent. The frequency of the different types thus depends upon "race" (see sec. 3.6516).

The characteristics M and N are inherited in a simple way, according to the Mendelian law. A single pair of genes controls the appearance of M and N; the gene M produces the characteristic M, the allelomorphic factor N produces N. In the heterozygote (MN) M and N appear together, so there is no definite dominance. The three serological types (phenotypes) correspond to three genotypes:

phenotypes:	"M"	"N"	"MN"
genotypes:	MM	NN	MN

The children to be expected in any mating may be derived very easily from the genetic formulas of the parents. This has important consequences for tests of paternity, as will be discussed below.

2.44 Technic of Demonstration

Immune sera from rabbits which have been injected repeatedly with human erythrocytes (usually group O) of the corresponding types ("M" or "N") are used. The routine of injection is the same as for the production of anti-A and anti-B (sec. 2.322). The diagnosis may be made in two different ways, of which only the first is important practically.

2.441 Detection Using Absorbed Immune Sera

Strong type-specific immune sera are needed, also control erythrocytes which lack the corresponding type characteristics. For example, in testing for the factor M we shall need N corpuscles as a control.

2.4411 Preparation of M and N Testing Fluids. To prepare a type-specific absorbed serum, the immune serum is diluted, and the species antibody removed by treatment with erythrocytes lacking the type characteristic. The completely absorbed serum should contain only the desired anti-M or anti-N antibody; it must be free from species agglutinin and other group or type antibodies.

The crude immune sera are diluted 1:15 to 1:30; we generally prefer 1:15 or 1:20. One cc. of each dilution is mixed with 0.5 cc. of thrice washed, packed erythrocytes of opposite type, M for serum anti-N, N for anti-M. For the production of anti-M and -N agglutinins only cells of

types OM and ON are used, but in absorption, cells of any group, O, A, B, or AB, may be used, so long as the blood does not contain the factor M, or N, as the case may be, against which it is wished to obtain agglutinins. A good plan is to mix cells (after washing) of types OM, AM, BM, etc.

If the immune serum is fresh, it must be inactivated (heated to 55° for 30 minutes) before being absorbed, to avoid hemolysis of the added cells. This is not necessary with stored serum.

The mixtures are allowed to stand at room temperature (the absorption may be carried out at 37°, but more cells may be required; it is sometimes desirable to try both methods with a given serum) for 30 minutes, then centrifuged and the supernatant tested, by the centrifuge technic, against cells of types M, MN, and N. If all the non-specific agglutinins have been removed from an anti-M serum, for instance, it should agglutinate bloods of the first two types and fail to agglutinate those of the last type. If N bloods are agglutinated, non-specific agglutinins still remain, and the serum must be absorbed further, with $\frac{1}{4}$ instead of $\frac{1}{2}$ the volume of cells. This is continued until the serum is completely specific. With anti-N sera, it is important not to carry the absorption beyond the point absolutely necessary, for continued treatment will eventually remove the anti-N agglutinins also (42, 43).

When the various diluted sera have each been completely absorbed, they are all tested together by the centrifuge technic against M, MN, and N cells. Some sera will probably be found too weak to use, or entirely negative. Rabbits apparently produce satisfactory anti-M sera in about one-third of the attempts, anti-N in about two-thirds (89).

A typical experiment showing the absorption and testing of several anti-N sera is shown in table 12. In this hypothetical case, serum 633 or 634 would be selected for final orienting absorption. Serum 634 requires less blood but 633 might be better if absorbed in a dilution of 1:20.

The strength of the absorbed sera can also be estimated by titration (90) but our experience indicates that this is not always worth the trouble. For ordinary purposes, the important thing is to have a testing fluid which reacts strongly under the conditions of the test. Sera are occasionally found which have a good titer but give poor agglutination; these are not desirable. Experience also suggests that the strength of agglutination of a serum may be on the whole a better indication of its probable keeping qualities.

We are now ready to consider the final orienting experiment. The best sera are selected, and diluted 1:10, 1:15, and 1:20. One cc. of each dilution is now absorbed as before, a record being kept of the amount of cells used. The absorbed fluids are compared, and depending on strength of agglutination and economy in cells, the dilution to be used in the prepara-

tion of larger amounts of testing fluid is selected. It is convenient to make the final preparation in lots of 20 or 50 cc. of diluted serum, depending on the amount desired, and the size of the centrifuge cups available. The absorbed sera are preserved by the addition of acriflavine (sec. 2.3223).

TABLE 12

TYPICAL RESULTS OBTAINED ON ABSORBING FIVE ANTI-N SERA

Serum diluted 1:15	Reaction with cells	Serum number				
		631	632	633	634	640
Before absorption	M	+++	+++	+++	+++	+++
	MN	+++	+++	+++	+++	+++
	N	+++	+++	+++	+++	+++
After first absorption (½ vol. M cells)	M	+++	+	++	+	+
	MN	+++	++	+++	+++	++
	N	+++	++	+++	+++	+++
After second absorption (¼ vol. M cells)	M	++	−	±	−	−
	MN	++	−	+++	++	±
	N	+++	+	+++	+++	+
After third absorption (¼ vol. M cells)	M	+	−	−		
	MN	+	−	++±		
	N	+±	−	+++		

Strength of agglutination observed after vigorous shaking of racks containing centrifuged tubes, containing 1 drop of absorbed serum, 1 drop of test cells (1%) and 1 drop of saline. The symbol +++ indicates maximal agglutination; all cells remain in one large clump, or two or three clumps. The strength of agglutination decreases in the order +++, ++±, ++, +±, to +, which indicates a weak positive reaction (a few small clumps visible to the naked eye), and ±, which is a doubtful reaction. The symbol − indicates absence of agglutination.

2.4412 Technic of the Tests. The blood to be investigated is tested with absorbed anti-M and anti-N to find if it is agglutinated by them; if it is, then the factor in question is present in the blood; if not, the factor is absent. The centrifuge technic described in secs. 2.251 and 2.25121 is used. Control cells of known types M, MN, and N should be tested simultaneously. When the accuracy of the results is particularly important, as in medico-legal cases, the unknown bloods should be tested with at least three anti-M and three anti-N sera.

2.442 Detection Using Direct Absorption of Immune Sera (76)

This method is needed only in doubtful cases ("weak types"), where it may occasionally be helpful. Again a type-specific immune serum is required, and erythrocytes which contain the factor in question. A

previously absorbed serum is preferable, for the experiment can in that case be carried out with less of the unknown blood, but crude unabsorbed serum can be used.

A suitable dilution of the serum is absorbed with the erythrocytes to be investigated. The absorption is repeated until all agglutinins for the unknown blood are removed. Then a test is made to find out whether the immune serum can agglutinate a known blood of the type in question. If good agglutination results, then the factor in question was lacking in the blood investigated; but if the known type-specific blood is not agglutinated, then the unknown blood contains the factor in question. If this connection it must be remembered that some immune antisera, in particular some anti-N sera, are much weakened by repeated treatment with corpuscles, even those not containing N. Such sera are of course not suitable for this work.

2.45 Sources of Error in M, N Tests

Somewhat more practice and skill are needed for these tests than for routine blood grouping, and they should not be entrusted to inexperienced persons. The most important points to be watched are: the *cell suspension* should not be too concentrated (cf. 90), (1 to 2 percent is best), the temperature should not be unusually low, the cell suspension should be fresh, adequate controls should be used.

Because of differences in the temperatures of absorption and testing, or because of other factors, some absorbed sera which are completely specific when newly absorbed may on subsequent occasions give non-specific reactions of greater or lesser intensity. This may sometimes be due to employing a different technic (e.g. slide and microscope) in the tests from that used by the person who prepared the fluids; also, there is some evidence that such non-specific agglutinins sometimes become stronger as the fluids age. Such reactions, with a good serum, are always weaker than true positive reactions, and are easily excluded by using proper controls. They have, however, sometimes misled self-styled "experts".

Cell suspensions in saline should always be tested the day they are made. They may sometimes be used after one night's storage in the icebox, but the reaction they give is nearly always distinctly weaker. If blood is to be transported, as by mail, it should be packed as described in sec. 3.3553. Blood mixed with Rous and Turner's solution (sec. 2.1) has also proved satisfactory.

2.5 Subgroups of Group A and AB; (Factors A_1 and A_2 etc.)

2.51 General

Individuals of group A can be separated into two types, depending on the absorptive capacity of their erythrocytes for the agglutinins in B serum

(table 13). The terminology we use was introduced by Landsteiner and Levine (44).

If from an anti-A serum there is removed by absorption, with suitable amounts of A_2 blood, enough agglutinin so that the A_2 is no longer agglutinated, the serum shows itself still active for blood corpuscles A_1. Such a treated serum can be used for differentiation of the two sub-groups (19, 39, 54). It also differentiates two subgroups of group AB (table 13).

TABLE 13

SUBGROUPS OF A AND AB

Group	Subgroups
A	A_1 A_2
AB	A_1B A_2B

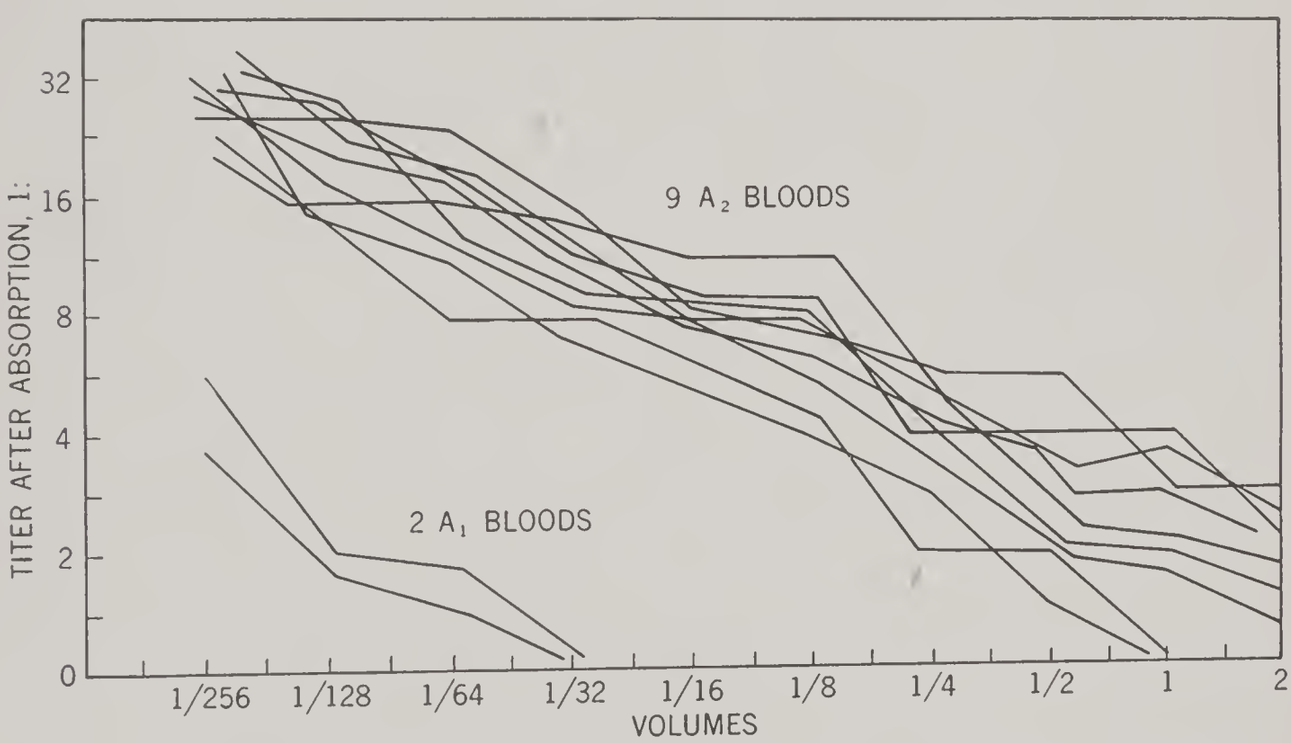

Fig. 15. Identification of A_1 and A_2 cells by titration (after Thomsen, Friedenreich and Worsaae).

The difference between the subgroups is especially well shown by determining, according to Thomsen, Friedenreich and Worsaae, the amount of blood corpuscles needed to absorb different amounts of agglutinin. This gives curves of the sort shown in figure 15. The blood of adults and older children can usually be placed in one of the families of curves without difficulty. (Especial care is needed with group AB). With children, especially very young children, a somewhat different procedure may be required.

2.511 Differences in the Agglutinogens

Cells of group A_2 are not completely devoid of absorbing action on anti-A_1 agglutinins in sera of group B. If larger amounts are used, effects on the agglutinating power of the serum for A_1 cells can be produced.

Conversely, absorption of B serum with very small amounts of A_1 cells has an effect similar to absorption with the usual amounts of A_2 cells (see table 14).

TABLE 14

THE VON DUNGERN AND HIRSZFELD PHENOMENON (25)

Group B serum, after absorption with	Blood cells	Dilution of serum, 1:						
		2	4	8	16	32	64	128
Unabsorbed	A_1	+++	+++	++	++	++	+	+
	A_2	+++	++	++	+	+	0	
$\frac{1}{8}$ vol. A_2 cells	A_1	++	+(+)	+	+	(+)	0	
	A_2	0	0					
$\frac{1}{256}$ vol. A_1 cells	A_1	++	+(+)	+	+	tr	0	
	A_2	0	0					

TABLE 15

LANDSTEINER-WITT PHENOMENON

ACTION OF AGGLUTININS IN SERUM OF GROUP B ON A_1 AND A_2 ERYTHROCYTES

Number of absorptions of serum	Amount of packed erythrocytes	Split off agglutinin		Titer vs.	
		Fluid number	Temperature	A_2	A_1
1	$\frac{1}{32}$ vol./A_2	1	ca. 20°	64	128
2	$\frac{1}{2}$ vol./A_2				
3	$\frac{1}{32}$ vol./A_1	2	ca. 20°	0	32
			0°	<2	64

That the difference between A_1 and A_2 cannot be merely quantitative, however, is indicated by the experiments of Landsteiner and Witt, on splitting off agglutinins from cells of the two types, summarized in table 15. The phenomenon of Landsteiner and Witt might be explained by assuming two qualitatively different anti-A agglutinins, α and α_1, and also two qualitatively different agglutinogens, A_1 and A_2. A_1 will react more strongly with α than with α_1, and A_2 reacts less intensely with α than A_1, and only feebly with α_1 (see figure 16).

A_2 cells have also been found to react more strongly with certain sera which affect most strongly cells of group O, and with certain weak agglutinins occasionally found in serum of group A_1B (see table 16). Thus, for the differentiation of the two subgroups certain bovine sera have been

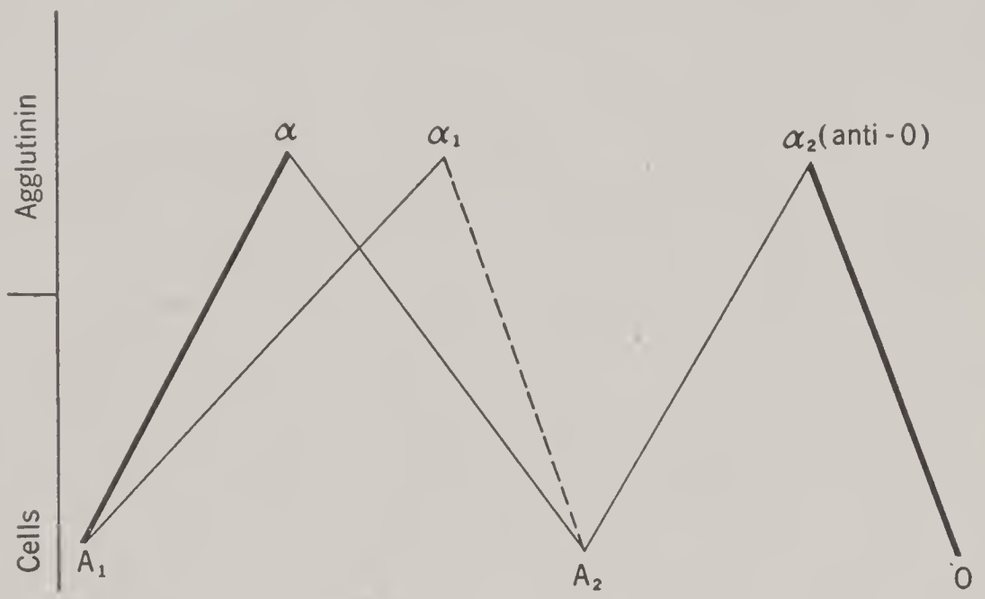

Fig. 16. Hypothetical relation of A_1, A_2, and α and α_1 (Friedenreich) (25, 90)

TABLE 16

REACTIONS OF A, B, AND AB ERYTHROCYTES

Cells	Group B serum, absorbed c. A_2	Reagent				
		Split off agglutinin		Certain A_1B (or A_1) sera (α_2)	Certain A_2B (or A_2) sera (α_1)	Certain cow* sera, abs. c. A_1 or A_1B (α_2)
		From A_2	From A_1			
O	−−	−−	−−	+	−− (or ±**)	++
A_2	−−	+	−−	+	−− (or ±**)	+
A_1	++	+	+	−−	+	−−
A_2B	−−			±	−− (or ±**)	±
A_1B	+			−−	+	−−

* And other animal sera. See table 17.

** At room temperature or lower.

found suitable. They are absorbed with human blood A_1, and they then act selectively on subgroup A_2 (table 17).

The difference between A_1 and A_2 does not correspond to the difference between the homozygote AA and the heterozygote AO, although it might be thought that the heterozygote AO, having but a single "dose" of A, would react less strongly with an anti-A serum and thus behave like A_2. To demonstrate this point it is sufficient to tabulate the ratio of A_2 to A_1

TABLE 17

OCCURRENCE OF ANTI-O AGGLUTININS* IN SERA OF DIFFERENT SPECIES (15)

Species	Number tested	Number usable	Number having titer 1:					
			4	8	16	32	64	128
Dog	335	16	7	4	4	1		
Guinea pig	140	0						
Cat	316	24	5	7	10	1	1	
Chicken	110	24	2	10	8	4		
Cow	211	34	2	9	12	7	1	3

* Agglutinins acting on O (and A_2) erythrocytes after the serum has been absorbed with A_1B erythrocytes (74).

TABLE 18

COMPARISON OF RATIO A_2/A_1 AND THE RATIO OF HETEROZYGOTES TO HOMOZYGOTES IN GROUP A

($AO/AA = 2pr/p^2 = 2r/p$)

People	Place	Investigator	A_2/A_1	AO/AA
Egyptians	Cairo	Boyd and Boyd	0.24	3.83
Irish	Dublin	Boyd and Boyd	0.46	8.00
Hawaiians	Hawaii	Nigg	0	3.16
Negroes	N. Y. City	Landsteiner & Levine	0.67	6.73

TABLE 19

TYPICAL RESULTS OF ATTEMPTS TO DISTINGUISH SEROLOGICALLY BETWEEN HOMOZYGOTE AND HETEROZYGOTE IN GROUP A (15)

Group	Genotype	Titer of cow serum no. 14 vs. O cells, after absorption with cells of the different groups, 1:					
		1	2	4	8	16	32
A_1	AA	+	+	+	+	+	±
A_1	AA	+	+	+	+	+	−
A_1	AO	+	+	+	+	−	−
A_1	AO	+	+	+	+	−	−
A_1	AO	+	+	+	±	−	−
A_2	AO	+	±	−	−	−	−
A_2	AO	+	−	−	−	−	−
Control (unabsorbed serum)		+	+	+	+	+	+

and of AO to AA (calculated from the frequencies of the groups) for certain populations (see table 18). Apparently the difference between the hetero-

zygotes (e.g. AO) and homozygotes (e.g. AA) can under some circumstances be distinguished by laboratory test, as is indicated by certain experiments of Dahr (15), using anti-O agglutinins contained in normal cow serum (see table 19). In this respect only, there is a connection between the two phenomena.

2.512 Differences in the Agglutinins

Our knowledge of the agglutinins in the subgroups of groups A and AB has recently been discussed by Wiener and Silverman, and this discussion is partly based on their paper (95). The serum of most group B individuals contains two varieties of anti-A agglutinins. One of these, designated as anti-A_1 or α_1, agglutinates blood containing agglutinogen A_1 but reacts hardly at all with A_2 bloods; the other, designated as anti-A or α reacts almost equally on bloods of both subgroups. The anti-A agglutinins in absorbed immune sera seem to be more homogeneous (92). Reagents for testing for A_1 may be prepared by treating suitable B sera with A_2 blood, thereby removing the common α agglutinin and leaving agglutinin α_1 behind. Bloods agglutinated by such absorbed B sera belong to subgroup A_1 or A_1B; bloods of groups A and AB not agglutinated by these sera belong to subgroups A_2 and A_2B, respectively, (90). The subgroups A_2 and A_2B are much more frequent in negroes than in whites (45).

While the reactions with α and α_1 agglutinins (table 15) point to a qualitative difference between agglutinogens A_1 and A_2, quantitative differences are seen in the reactions with natural and immune anti-A sera. In general, A_1 is more sensitive to agglutination that is A_2; moreover, the reactions of AB bloods with anti-A sera are weaker than those of bloods containing agglutinogen A alone. The relative sensitivity of the A agglutinogen in the four subgroups can be indicated as follows: $A_1 > A_1B > A_2 > A_2B$.

A small percentage of group A and group AB individuals have in their sera irregular agglutinins reacting with bloods of the opposite subgroup, as was first pointed out by Landsteiner and Levine (44). These occur most frequently among persons in subgroup A_2B; according to Wiener as many as one-fourth to one-third of such bloods contain agglutinin α_1. The agglutinin α_1 is far less common in subgroup A_2 individuals that in subgroup A_2B, and agglutinin α_2, which can occur only in A_1 and A_1B bloods, is found only rarely. The irregular agglutinins α_1 and α_2 are usually very weak, giving no reactions at all in vitro at body temperature. The so-called agglutinin α_2 has the peculiarity of reacting even more strongly with group O blood than with A_2 blood. For this reason it is also designated as an anti-O agglutinin.

2.513 Hypothetical Explanations

A number of hypothetical explanations of the behavior of these subgroups have been offered. They are summarized in table 20.

TABLE 20

VARIOUS HYPOTHETICAL ANTIGENIC STRUCTURES OF SUBGROUPS A_1 AND A_2 (5)

Cells of subgroup	Antigenic structure				
A_1	AA_1	A_1 or AA_1	yA	FyA or FF′yA or y′FF′yA	AAAA or AAAO or AAOO
A_2	A	A_2 or AA_2	xA	xA or F′xA or x′FF′xA	AOOO
reference	(13, 19, 29, 54)	(54, 40)	(56, 83)	(84)	(62)

A represents a hypothetical common antigen, A_1 and A_2 two related but different antigens, O a hypothetical O agglutinogen, F a certain group of Forssman-like antigens. y represents a measure of quantity, more than x.

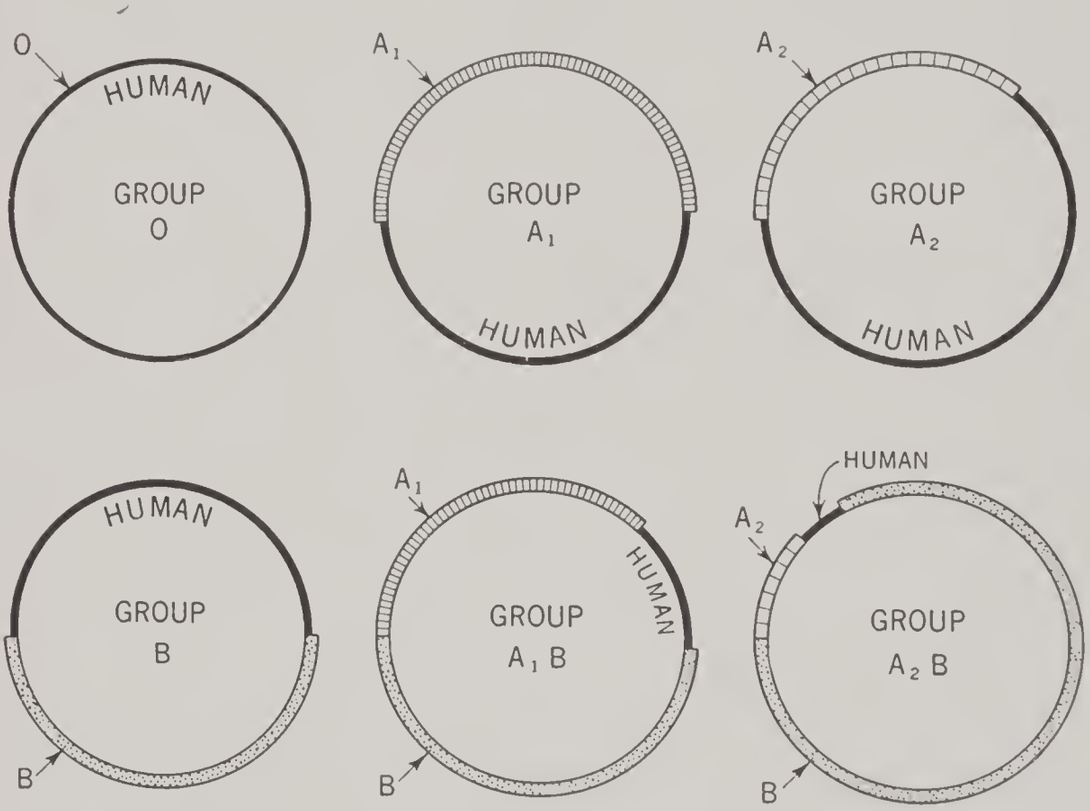

Fig. 17. Hirszfeld's theory of the antigenic composition of A_1, A_2, and A_1B and A_2B erythrocytes (5).

Hirszfeld (31) has proposed a hypothesis which is presented diagrammatically in figure 17. Matta (62) has reported results which on the whole agree with such a picture.

2.514 Heredity

As was first shown by Landsteiner and Levine (45, 41), the agglutinogens A_1 and A_2 are hereditary. Thomsen, Friedenreich and Worsaae (85, 86) presented evidence which indicated that the six groups, O, A_1, A_2, B, A_1B and A_2B are inherited by means of four allelomorphic genes, A_1, A_2, B and O, where A_1, A_2 and B are dominant over O and A_1 is dominant over A_2. The heredity of the subgroups has been discussed in detail by Wiener (90, 94).

2.52 Technic of Differentiation of Subgroups of A and AB

2.521 Location of Donors of Subgroups A_1 and A_2

If individuals known to belong to these subgroups are not available in the laboratory, and suitable reagents are not at hand, suitable donors for blood in further experiments may be located as follows:

Test a large number (20 to 30) of bloods of group A simultaneously, with several not-too-strong B sera, or, even better, with absorbed immune anti-A sera. Several of the A bloods will be found to give distinctly weak reactions. These probably belong to subgroup A_2. This surmise may be verified by using cells of these bloods to absorb B sera as described below, and by the use of the reagents thus produced the rest of the A bloods may be classified.

It has also been observed that some fresh B sera will hemolyze A_1 cells and agglutinate only A_2 cells (44).

2.522 Preparation of an Anti-A_1 Testing Fluid

For this a good human serum of group B is used. Not all B sera are suitable; a satisfactory serum can be chosen only by experiment.

The serum is mixed with $\frac{1}{3}$ to $\frac{1}{4}$ its volume of packed, thrice-washed cells of subgroup A_2 and allowed to stand 30 minutes at room temperature. Usually it will be found that the fluid obtained after centrifuging this mixture agglutinates A_1 bloods, but not A_2 bloods. If there is still distinct agglutination of A_2 cells, the absorption may be repeated, but with a smaller quantity of cells. These absorbed B sera do not retain their strength as well as untreated sera.

Immune absorbed anti-A sera from rabbits injected with human A cells or sheep cells can also sometimes be rendered specific for A_1 cells by absorption with A_2 erythrocytes, but are usually less satisfactory (37, 6).

2.523 Preparation of an Anti-A_2 (O) Testing Fluid

Sera from 10 to 15 cattle are inactivated and absorbed as above, but using washed packed erythrocytes of subgroup A_1, or preferably A_1B. One or more sera reacting with group O and subgroup A_2 will usually be obtained.

2.524 Differentiation of the Subgroups

Bloods of group A are tested, by the centrifuge technic, with the anti-A_1 and -A_2 reagents just described. Bloods of subgroup A_1 should be agglutinated by the first and not by the second, whereas the reverse should be true of the A_2 bloods. Of the two reagents, the anti-A_1 seems on the whole the more reliable.

Occasionally doubtful bloods will be found, which either react with both reagents, or fail to react with either. Such bloods can often be diagnosed by setting up tests with the testing fluids diluted successively 1:2, 1:4, etc., with known bloods of subgroup A_1 and A_2 as controls. There is usually a sharp difference in titer which makes a decision possible (figure 15). For example, an A_2 blood might be agglutinated by the anti-A_2 serum diluted 1:4 but fail to be agglutinated by the anti-A_1 diluted 1:2 (cf. 37).

If the investigator is fortunate enough to find donors of subgroups A_1 and A_2, or A_1B and A_2B, whose sera contain the irregular agglutinins anti-A_2 and anti-A_1, these will prove useful in differentiating the subgroups (62).

Anti-O sera reacting well with A_2 have also occasionally been produced by absorbing with A_1B blood sera of rabbits injected with O cells.

Ponsold (68, 69) has proposed a micro-technic for measuring the absorptive power of A cells as a means of differentiating the subgroups. For discussion of other methods, the reader is referred to (68, 69, 17, 37).

2.525 A_3 and A_4

If immune anti-A, or the strongest human B sera are used, there is little difference in the strength of reaction of A_1 and A_2 bloods. But Fischer and Hahn (23) and Friedenreich (26), reported independently that individuals of group A exist whose bloods react feebly even with the most potent anti-A sera. In parallel tests where A_1 and A_2 cells show complete agglutination, cells of subgroup A_3 will be only partly agglutinated, about half of the cells remaining free (95). Among 5000 individuals examined, Friedenreich found only 6 A_3 bloods.

The weak reaction of A_3 bloods is said to be quite characteristic (27). Friedenreich explained this phenomenon by postulating a third sort of A agglutinogen, designated by him as A_3, and giving rise to two additional subgroups, A_3 and A_3B, respectively. The property A_3 is hereditary, since an additional 46 A_3 and A_3B individuals were found by Friedenreich by testing the 260 relatives of the 6 individuals found in his first study. He suggests that the agglutinogen A_3 is transmitted by means of a fifth allelomorphic gene, A_3. Gene A_3 is considered to be recessive to genes A_1 and A_2 but dominant over gene O.

Gammelgaard and Marcussen, in the course of examining 60,000 bloods (27) especially with an eye to detecting weak A reactions, found one blood

which reacted much more weakly than A_3. It gave a doubtful reaction even with B sera having a titer of 1:64 to 1:128, but could be detected by using the rare B sera which have a titer of about 1:1000. According to these authors, it is easy to distinguish the reaction of A_4 from that of A_3.

Bloods of subgroup A_3 and A_4 apparently are likely to have the irregular agglutinin anti-A_1 (secs. 2.264 and 2.512) in their serum.

2.53 Subgroups of B

Numerous workers have of course attempted to show analogous differences in group B, by absorbing A sera with a variety of B bloods and testing against others. For example we may mention the work of Worsaae (99). Most of these attempts have been unsuccessful. However, positive results have apparently been obtained by some workers (34, 32, 64, 62). The differences between B_1 and B_2 do not seem to be as pronounced as between A_1 and A_2. Our knowledge of them, summarized by Matta (62), is not yet sufficient to allow their application in forensic work.

2.54 Subgroup MN_2

In the present state of our knowledge, and in view of the rarity of the N_2 factor, the chief concern of the investigator will be to avoid mistaking MN_2 bloods for M bloods, since in the presence of M, N_2 reacts especially weakly. This can probably be assisted in most cases by using only strong anti-N sera known to give good typical reactions, always including bloods of type M, MN, and N as controls, and whenever, as in certain cases of disputed paternity, it is important to distinguish between MN_2 and M, retesting the bloods with several powerful anti-N sera. As a control, MN cells which have been washed repeatedly with saline to weaken their reactivity, may be used.

Langenberg (55) was successful in detecting N_2 by the use of anti-N sera which had been concentrated after absorption, but such sera keep very poorly. Pietrusky has employed tests on slides at a temperature of 5° (67, 66).

2.6 Technic of Detection of Other Factors in Human Blood

The reader may refer here to the general remarks we have made upon the use of immune sera and the M, N tests (secs. 2.3 and 2.41). The only factors, other than the A, B, O and M, N series, which seem likely to have practical importance in the immediate future are P and Rh, and we accordingly confine our attention to them.

2.61 The P Factor (42)

Anti-P agglutinins occur in the sera of certain animals, such as rabbits, hogs, cats, and cattle (41, 51, 77). According to Dahr, Offe and Weber

(16) hog and horse sera are particularly useful. These authors prepare an anti-P reagent as follows:

Several crude inactivated sera are treated with a mixture of washed, packed erythrocytes of P-free individuals of groups O, A, and B, and allowed to stand at 5° for two hours. The amount of packed cells required is judged by preliminary test of the sera against P-free and P-containing bloods. Sera are suitable which now agglutinate P+ bloods distinctly and do not agglutinate P− bloods. Occasionally a second absorption with a small volume of P− cells is necessary.

Although it is difficult, anti-P sera have been produced by absorbing the sera of rabbits injected with P+ blood.

If individuals of types P+ and P− are not available, according to Wiener (90), the factor P may be identified by absorbing a number of different animal sera with a number of human bloods, until reagents are obtained which give distinct and parallel reactions, independent of A and B, with certain human bloods. These sera will contain anti-P agglutinins.

Landsteiner and Levine (42) concluded that the property P is not a single, well defined entity, but a group of related agglutinogens, since results obtained with anti-P agglutinins obtained in different ways do not always run exactly parallel, and some sera give "graded reactions".

2.62 The Rh Factor

2.621 The Agglutinogen Rh

Landsteiner and Wiener (52) found that the sera of certain rabbits which had been immunized with the blood of the rhesus monkey, when absorbed with certain human bloods, designated as Rh−, agglutinated other human bloods, designated as Rh+.

Attempts to produce suitable antisera by injecting Rh+ human bloods into rabbits have apparently not always been successful. Landsteiner and Wiener (53) recommend the use of diluted, unabsorbed serum from guinea pigs which have been injected with rhesus blood. Levine (57a) prefers the use of human anti-Rh agglutinins.* The actual tests are much the same in either case.

The technic of Landsteiner and Wiener will be given in their own words:

"For the production of the sera large guinea pigs were injected intraperitoneally with a suspension of washed red cells of rhesus monkeys, each animal receiving a dose corresponding to 1 cc., in later experiments to 2 cc. of whole blood. The injection was repeated after 5 days and 1 week later the animals were bled. The sera of the majority of animals were found to

* Interference by regular isoagglutinins in such sera can be avoided by neutralizing them by the previous addition of saliva from (secreting) persons of group A and/or B (91).

show a difference between the two sorts of blood, Rh+ and Rh−, and in a group of ten animals usually one or more were found that yielded sera suitable for practical diagnosis. The manner of selecting the sera is given below.

While in the case of the immune rabbit sera the reagent was prepared in the customary way by absorbing the diluted serum with negatively reacting blood, it was found with several guinea pig sera that absorption with human blood resulted merely in a non-specific diminution of the agglutinin content, no matter whether positive or negative blood was used. This led us to test the effect of simple dilution, and indeed it was found that a distinction between positive and negative bloods could be made directly without absorbing the sera. (As an analogy, mention may be made of rabbit immune anti-A sera which cannot be specifically absorbed with A_2 cells to produce a reagent for A_1, absorption with A_2 blood serving merely to diminish the agglutinin titer.)

The method for determining suitable sera consists in making serial dilutions by halves and testing with known negative and positive blood. Those sera which show in three (or more) successive dilutions negative reactions with the former and positive ones with the latter blood are usable.

The actual tests can usually be carried out simply by selecting a dilution of the serum, e.g., 1 to 10, which gives no reactions with negative but definite reactions with positive bloods, those sera that contain appreciable amounts of anti-A or anti-B agglutinins having been previously absorbed with small quantities of A and B blood. Since the sera are used diluted, inactivation is mostly unnecessary. The blood to be tested should be fresh.

Another method, alternative to dilution alone, is to absorb the sera diluted, e.g. 1:4, with a quantity of blood (using A or B cells if indicated) sufficient to remove the reaction with Rh-negative blood.

Two drops (0.1 cc.) of the test fluid are then mixed with one drop of 2 per cent (in terms of blood sediment) washed blood suspension, freshly prepared, in a narrow tube of 7 mm. inner diameter, and allowed to stand at room temperature. Readings are taken after sedimentation has occurred, usually after 30 minutes to 1 hour, by direct inspection of the bases of the tubes, with a hand lens. Negatively reacting bloods then show a circular deposit with a smooth edge, while positive bloods have a wrinkled sediment with a serrated border or show a granular deposit. From these readings, as a rule, the diagnosis can readily be made. The readings are facilitated by using racks having small holes beneath the bottoms of the tubes. Following the reading the tubes are shaken and the

sediment examined after it forms again. A further examination is made after 2 hours, again inspecting the sediment. The tubes are then gently shaken and the suspension is examined microscopically: the negative blood samples are mostly perfectly homogeneous; the positive ones show various degress of agglutination, not infrequently visible to the naked eye. At times, the clumping is quite weak in spite of a distinctly positive sediment picture. Needless to say, positive and negative control bloods should be included in each test."

2.622 Detection of Anti-Rh Isoagglutinins

Agglutinins found in the sera of Rh− individuals who have been transfused with Rh+ blood seem to behave on the whole as "cold" agglutinins (93). Those found in the bloods of Rh− mothers following isoimmunization by an Rh+ fetus seem to behave as "warm" agglutinins (58). Since their detection may be important in the investigation of reactions following transfusion (sec. 3.18) we give the technics of detecting these agglutinins in these authors' own words. It is not yet certain which of these methods, if either, is preferable for routine use. In some cases where transfusion reactions are due to Rh incompatibility, no anti-Rh agglutinins seem to be detectable by either method.

Levine, Katzin, Vogel and Burnham (59) state: "It is therefore recommended that in performing the compatibility test in instances where isoimmunization may be a factor (in patients after repeated blood transfusions or in pregnancy cases, especially those women having repeated spontaneous abortions or babies with erythroblastosis fetalis) the mixture of patient's serum and prospective donor's cells be incubated for 15–30 minutes at 37° in a small test tube. This is then centrifuged for one minute at low speed (500 RPM) and the resuspended sediment is examined microscopically for the presence or absence of agglutination."

Wiener and Peters (93) state: "1. Two drops of patient's serum, preferably separated from the clot at refrigerator temperature, are mixed wit one drop of donor's cell suspension in a small test tube.

2. In a second tube a similar mixture of patient's serum and patient's cells is set up.

The tubes are placed in ice-water for 5 minutes, then centrifuged while still cold and the mixtures gently shaken. The reactions are read macroscopically and microscopically. If neither tube shows a reaction the donor is compatible. If both show a reaction, we are dealing with an autoagglutinin and the donor probably can be used without danger. If tube 1 shows agglutination and tube 2 does not, the donor is incompatible and others must be tested in order to find a suitable one."

2.7 Investigation of Material Other Than Erythrocytes

2.71 Test for Agglutinins

The examination of fresh sera for agglutinins has already been discussed (sec. 2.251). In some cases they may be demonstrated also in dried blood (47). For tests on pericardial fluid, see sec. 3.4231.

2.711 Lattes' Cover Glass Method

The serum agglutinins of dried blood are conveniently detected by the cover glass method of Lattes (56) (sec. 3.1185). Since the agglutinins in the material may have become weaker, especially sensitive test erythrocytes should be employed. In making the test, pseudoagglutination must be especially looked out for. Lifting and replacing the cover slip, or simply slight pressure on it, is usually sufficient to abolish the pseudoagglutination which may occur.

A parallel test with red cells of group O is indispensable for a control. If these corpuscles also seem to be agglutinated, then it is possible that the agglutination observed in the main experiment was also really pseudoagglutination.

Care must be taken to put only very small particles of dried blood on the slide, for otherwise the cover slip will be raised too much. The very small amount of material needed is an advantage for medico-legal work. The agglutinins, if they are present, may be readily detected in a particle of dry blood weighing about 0.1 mg. The procedure to be followed in the experiment depends somewhat on the condition of the material. If little crusts and scales may be scraped off of the substrate, as with metal, glass, stone and leather, they may be investigated directly. If larger amounts of blood are available, extracts may be made which can be tested in the test tube. The test for agglutinin content is carried out in small test tubes as described above.

It is preferable not to make the reading in the presence of the usually dark colored and somewhat cloudy extracted fluid, but after allowing time for complete reaction (30 to 60 minutes) to centrifuge and replace the fluid by physiological saline. Then the sediment is shaken up and the reaction read.

2.72 Absorption Reaction

2.721 Binding of Agglutinins

The agglutinable substance can no longer be detected in dried blood by the direct agglutinin test, since for agglutination freely suspended corpuscles still retaining their shape and still unhemolyzed are required. Therefore, it is necessary to test the power of the agglutinable substance to bind the specific agglutinin.

When red cells are agglutinated by a group-specific serum, they are coated with combined agglutinin. This can easily be demonstrated by

separating the cells and supernatant fluid by centrifuging, and testing each separately. The supernatant now agglutinates new cells either weakly or not at all, whereas by appropriate treatment antibody can be recovered from the erythrocytes. The erythrocytes have "bound" the agglutinin. This specific power of combination is displayed not only by fresh, unaltered cells, but also by old dried cells, which can no longer be agglutinated. Therefore the test for agglutinin-combining power is an important procedure in the group diagnosis of cells which are not agglutinable, finding special application in the study of blood stains. Other tissues, for example kidney, salivary gland, and stomach wall, may be tested in this way.

2.722 Test of Unknown Material for Absorption of Agglutinin

The material to be investigated, in a finely divided state, if possible a fine powder, is mixed with anti-A and anti-B agglutinating serum. After a certain time of contact, the material is centrifuged off and the supernatant fluid is tested for its content of agglutinin. If the material under investigation contains an agglutinable substance corresponding to one of the agglutinins, then the supernatant fluid will be very much weakened.

2.7221 Reagents. The preparation of the proper reagents is probably the most important step in the procedure. It would be even less appropriate in this work than in clinical serological tests to make use of unknown or improperly tested reagents. The reagents required are sera which react with the agglutinogens A and B. It has been our practice for some years to make use of immune anti-A and anti-B sera in such work, as well as of human sera of types B and A.

This enables us to incorporate in every set-up a valuable check on the specificity of the reaction, and also helps to guard against errors due to accidental exchange of sera or of cells in setting up a test. It will readily be appreciated that the demonstration that a stain has the power of removing a certain type of agglutinin, whether this is of human or immune animal origin, strongly supports the conclusion that the reaction is specific, and thus to be relied on. It is desirable to employ for such experiments A serum (anti-B) and B serum (anti-A), with a known and roughly equal content of agglutinin. Sera of group O, which contain both the agglutinins anti-A and anti-B, are not always so satisfactory, because the titer of the two agglutinins is often quite unequal. Another argument against the use of O serum is the possibility that in the case of some O sera, the removal of one agglutinin may involve some removal of the other (54, 30).

The strength of the sera must be estimated by titration, by making progressively doubled dilutions, which are tested under the same conditions as those under which the sera will be used. Since the amount of material available for investigation is likely to be limited, this generally means it is best to carry out the tests in "well slides" or by the hanging drop method, on cover slips sealed with vaseline on little chambers on slides. In the

latter case, one small loopful of serum is mixed with one of A_1 erythrocyte suspension (ca. 1 per cent), and another mixed with B erythrocytes. The erythrocytes should be from individuals whose cells have been shown to react properly. After 5 to 15 minutes the reactions are read under the low power of the microscope. The reactions should be recorded according to a consistent system.

2.7222 Outline of Test. *Antisera.* Prepare mixtures of:

(1) Serum A (anti-B), 0.4 cc.; physiological saline, 1.6 cc., making 2 cc. of a 1:5 dilution.

(2) Serum B (anti-A), 0.4 cc., plus 1.6 cc. of physiological saline, also making 2 cc. of a 1:5 dilution.

Absorbed immune sera anti-A and anti-B are similarly prepared. If sufficient material is available, the test is carried out with each serum separately, but if the material is scanty, it is desirable to mix the two test sera and allow the unknown material to act on each mixture. When possible, human A and B sera are not mixed on account of the possibility of some neutralization of the agglutinins by A and B substances normally occuring in the serum. But if the material is very limited, and the A and B sera are now of equal strength, dilutions (1) and (2) are mixed, giving 4.0 cc. of a 1:10 dilution of anti-A and anti-B. The immune sera are similarly mixed.

Corpuscles. We may use washed red cells of group A as material for a model experiment. Since the agglutinogens A and B occur, in individuals of the appropriate groups, in practically every cell and fluid of the body, this method is by no means restricted to blood, however.

Combination. To the packed cells from 5 cc. of a 5% suspension is added 2.0 cc. of the diluted serum mixture. The resulting mixture is well shaken and allowed to stand 1 to 24 hours in the cold, then the supernatant is removed (figure 18).

The supernatant is tested (figure 18), along with the untreated serum mixture, for its content of agglutinins against cells A and B. Since we have plenty of material, we may use the test tube method in this case.

In test tubes 1 and 2 there is placed about 0.2 cc. of the supernatant, in tubes 3 and 4 about 0.2 cc. of the untreated serum. A cells are added to tubes 1 and 3, B cells to tubes 2 and 4 (1 drop of an approximately 5% suspension). The adjoining scheme shows the results for the special case of blood cells of group A.

Tube	Reagents	Agglutination
1	Treated serum + A cells	−
2	Treated serum + B cells	+++
3	Untreated serum + A cells	+++
4	Untreated serum + B cells	+++

The absence of agglutination in tube 1 shows that the agglutinin anti-A has been completely removed from the serum mixture by the corpuscles. Tube 2 shows that this combination was specific. The positive agglutination in tube 2 shows that the other agglutinin has not been affected. Any non-specific adsorbing agent such as animal charcoal would most probably have weakened both agglutinins to the same degree. AB cells would specifically remove both agglutinins.

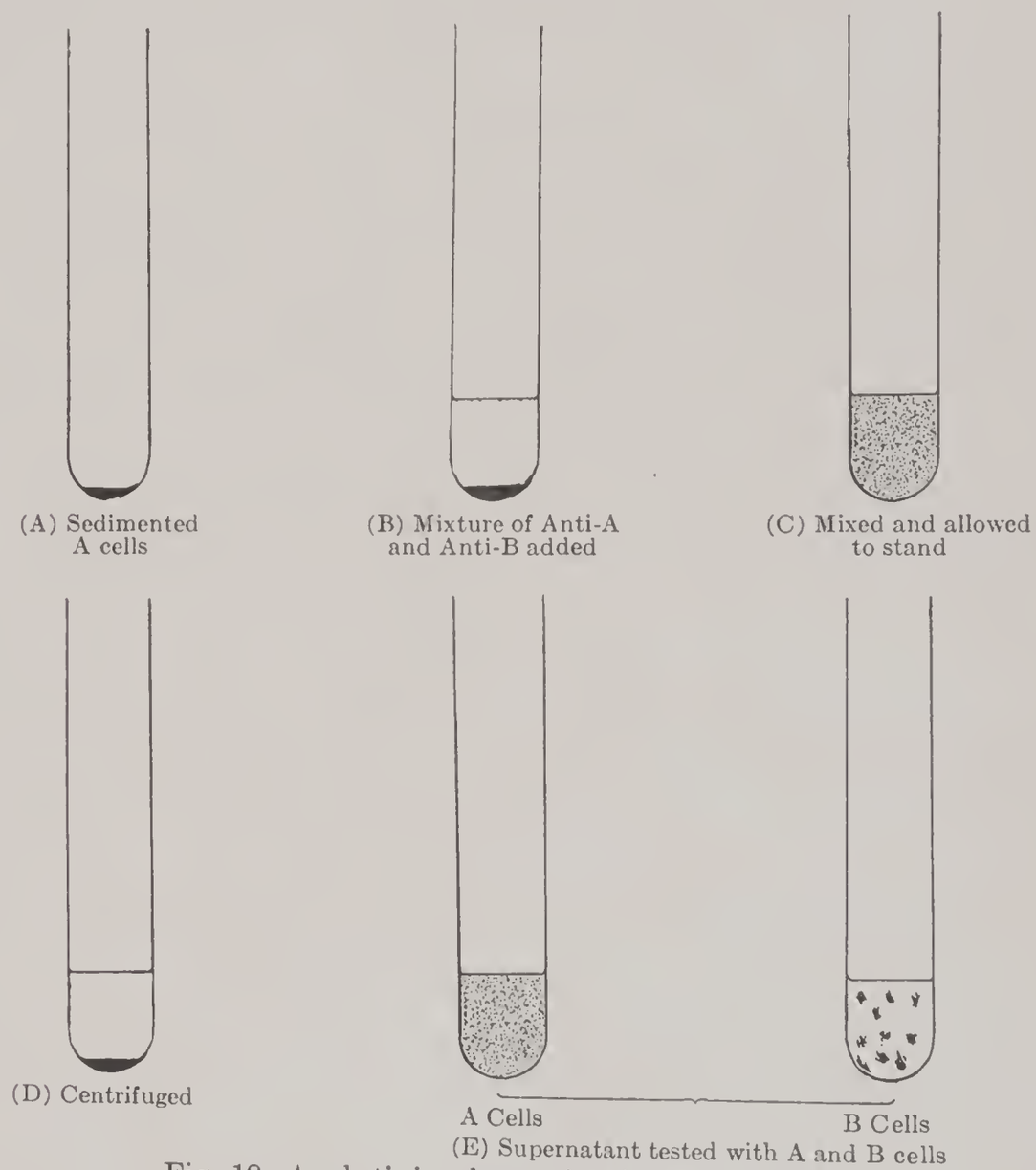
(A) Sedimented A cells
(B) Mixture of Anti-A and Anti-B added
(C) Mixed and allowed to stand
(D) Centrifuged
A Cells
B Cells
(E) Supernatant tested with A and B cells

Fig. 18. Agglutinin absorption in test tubes

If a much smaller amount of the corpuscles had been taken for the experiment, the absorption of agglutinin would have been incomplete. Comparison of the treated and untreated serum mixtures would have indicated roughly the degree of removal. A slight weakening of the agglutination does not necessarily indicate specific removal of agglutinin.

With older material which might be mixed with unknown substances, attention must be given to the possibility of agglutinin removal by non-specific adsorption. Only a strong removal of antibody will be considered

significant. In some circumstances this can be checked by testing whether or not the material alone has a non-specific power of combination with other antibodies.

Controls, consisting of similar human material of known group, should be tested with the same sera, along with the unknown material. The behavior of the unknown should be evaluated by comparison with the behavior of the controls.

In carrying out such an experiment in actual practice, the quantitative relations must be kept in mind. Weakening of an agglutinating serum by absorption is easier, the weaker the agglutinin is. Therefore, when only a little material is available, *small* amounts of serum or diluted serum are used. However, non-specific weakening occurs most easily when small amounts of agglutinin are being used. Therefore, the more agglutinin is present, the more indicative of the presence of an agglutinable substance is a lowering of the agglutinating power of the serum.

In the most accurate work, the strength of each agglutinin is determined at the end of the experiment by titration.

In testing for remaining agglutinin in inhibition and absorption experiments, it is probably desirable not to use too strong cell suspensions, and particularly important to test the *absorbed* and *untreated* serum with suspensions of the same strength. Lund (61) has found that use of erythrocytes in a final concentration of 0.06 per cent gives 8 times the sensitivity obtained with suspensions of 0.5 to 1.0 per cent. Methods of making the tests with a volume of serum relatively large compared to that of the erythrocytes (centrifuging and removing most of the liquid), and use of well-slides (sec. 2.1, sec. 3.65233) have proved of value (61, 10).

2.73 Splitting off Bound Agglutinins (38)

The absence of agglutination in tube 1 in the above experiment shows the absorption of anti-A by the cells used. This absorption may be partially reversed by suitable procedures; most easily by careful warming. Recovering an agglutinin by splitting it off serves to confirm an absorption experiment, and can thus have diagnostic value. In some circumstances, such as in a criminal case, it is particularly desirable to confirm an important finding by several procedures.

To carry out such an experiment, the sediment from the corpuscle-serum mixture may be used. If sufficient material is available, however, it is more desirable to use a larger amount of cells and strong undiluted serum. About 2 cc. of packed red cells are mixed with an equal volume of a serum containing the corresponding agglutinin. The mixture is allowed to stand in the icebox for 2 to 24 hours, with occasional shaking. Then it is centrifuged, and the sediment washed three times with adequate amounts of saline. Then there is added to the sediment as small an amount as will

suffice (say 0.5 cc.) of ice cold saline. The mixture is centrifuged, and the supernatant tested for agglutinin. If the previous washing was sufficient, the supernatant will be found to be completely free of agglutinin. If agglutinin is found, the washing is repeated once or twice.

Then there is added to the sediment another small amount of saline and the mixture is well shaken. The real splitting-off experiment ("Absprengungsversuch") now begins (figure 19). The tube is placed for 5 minutes in a water bath at 54° and then centrifuged very quickly. To avoid cooling, the centrifuge cup is filled with water at 54°. The supernatant is poured off and tested for agglutinins against A and B cells.

The detection of bound agglutinin by splitting off is easier, the more agglutinin was taken up by the agglutinable substance, if care is taken to remove all of the mechanically adhering serum.

2.74 Sources of Error in the Absorption and Splitting-off Experiments

2.741 Absence of Combination Due to Deterioration of the Agglutinin

The basic rule is to consider only a *positive* result significant. However, failure to find A and B in dried blood in which the agglutinins anti-A and anti-B have been demonstrated will be a significant confirmation.

2.742 Confusion of Specific Removal with Non-specific Absorption or Inhibition

This source of error may usually be considered to be excluded if the blood in question has strongly decreased the strength of only one of the two agglutinins anti-A and anti-B, while the other has remained (quantitatively) unchanged, especially if the mixture of immune sera is similarly affected. Knowledge that the two agglutinins used are of about the same strength is, of course, a prerequisite for such a conclusion.

2.743 Failure of the Splitting-off Experiment

Whether a splitting-off experiment will succeed depends on the amount and the state of preservation of the agglutinogen. Failure is common. It should not be considered a contradiction of a successful absorption experiment. If successful, however, it may serve to confirm the results of absorption experiments.

2.75 Specific Inhibition

2.751 Principle

This procedure is related in principle to absorption. If to a group-specific serum there is added the specific antigen in dissolved form, usually there is no visible reaction, but the antibody is attached to the antigen molecules, and now no longer reacts with added red cells.

Agglutination of A cells by human anti-A agglutinin, or of B cells by anti-B is inhibited by antigen solutions; for example, by extracts of red

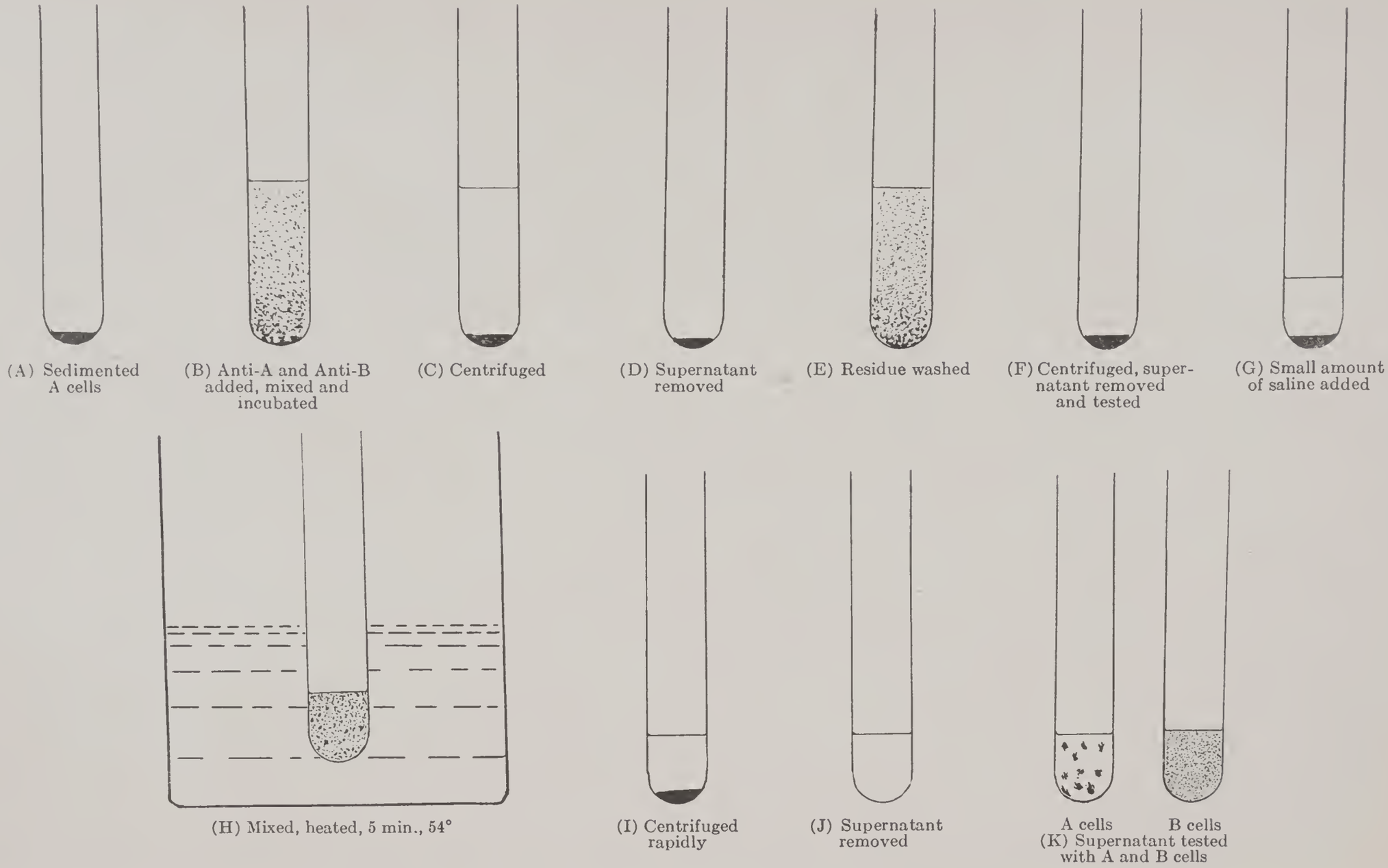
(A) Sedimented A cells
(B) Anti-A and Anti-B added, mixed and incubated
(C) Centrifuged
(D) Supernatant removed
(E) Residue washed
(F) Centrifuged, supernatant removed and tested
(G) Small amount of saline added
(H) Mixed, heated, 5 min., 54°
(I) Centrifuged rapidly
(J) Supernatant removed
A cells
B cells
(K) Supernatant tested with A and B cells

Fig. 19. Splitting off of bound agglutinin

cells and of organs. Antigen in solution is found in spermatic fluid, saliva, gastric juice, *succus entericus*, bile, urine, etc. The principle is illustrated by figure 34. It is important to keep in mind that 10 to 20% of all individuals do not show their blood group substances in the urine or in the gastric secretions.

2.752 Reagents

The inhibition reaction is best carried out with normal iso-agglutinins, for the dissolved antigens in tissue fluids do not react so readily with im-

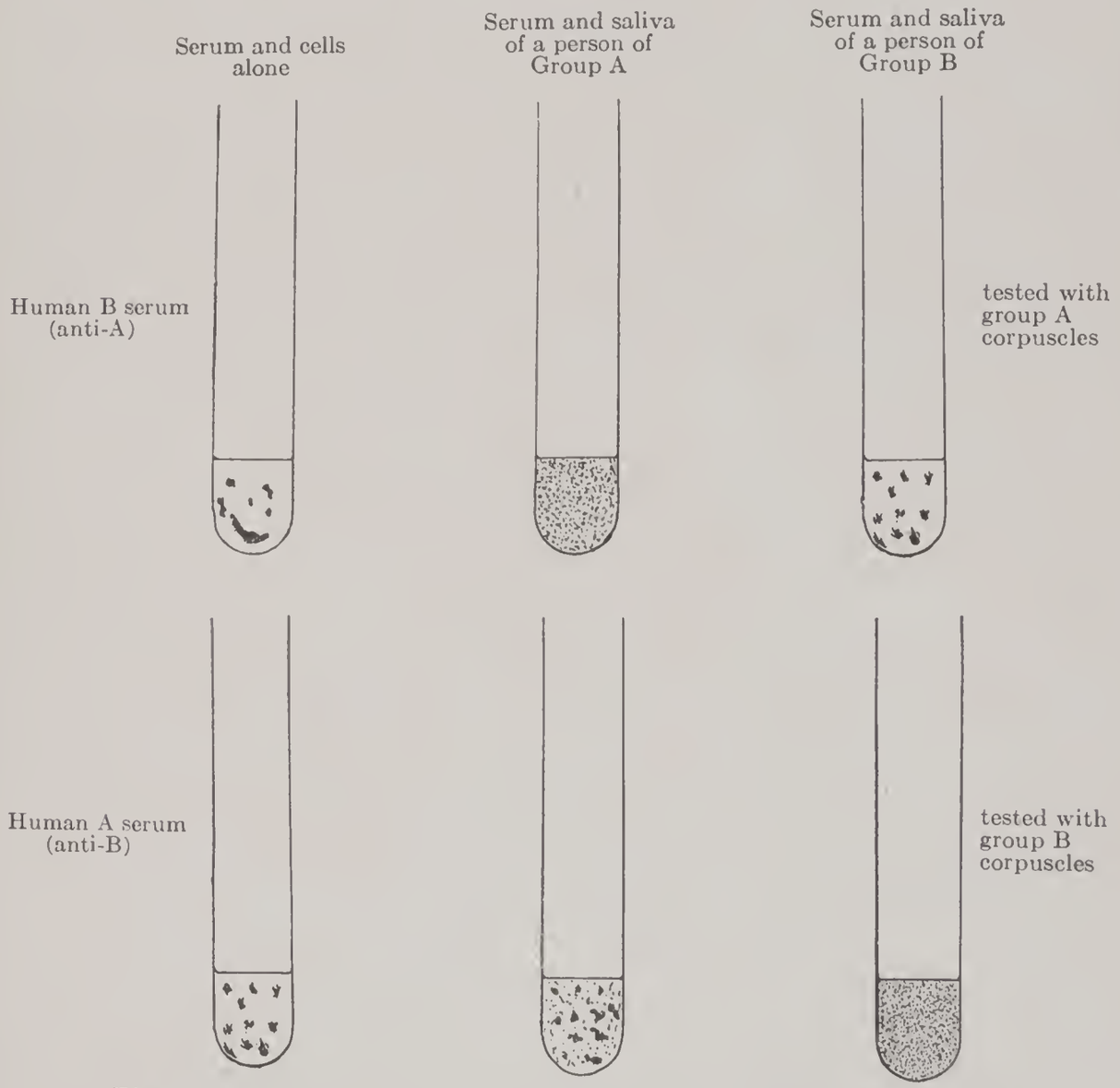
Serum and cells alone
Serum and saliva of a person of Group A
Serum and saliva of a person of Group B
Human B serum (anti-A)
tested with group A corpuscles
Human A serum (anti-B)
tested with group B corpuscles

Fig. 20. Scheme of the inhibition test (with saliva as an example)

mune agglutinins. In the case of A substance the sheep hemolysin (sec. 2.754) obtained by injecting rabbits with human corpuscles of group A, is also useful, being particularly sensitive. An exact titration of the antisera is essential. An antibody concentration which is not too great, but which still agglutinates strongly, is used. As an example of the technic, two tests on saliva of group A and group B will serve (figure 20).

2.7521 Preliminary Titration of the Iso-agglutinins. To increasing dilutions of serum there are added equal volumes of blood corpuscle sus-

pension (about 0.1 cc. of serum dilution and 0.1 cc. of cell suspension). Typical results are shown below:

Reagents		Serum dilutions						
Serum	Cells	1:5	1:10	1:20	1:40	1:80	1:160	1:320
anti-A	A	++++	++++	++++	+++	++	+	−
anti-B	B	++++	++++	+++	++	+	−	−

On the basis of these titrations, the anti-A serum is diluted 1:20, the anti-B 1:10.

2.753 Main Experiment: Inhibition Test

To each of a series of small test tubes is added 0.1 cc. of anti-A serum diluted 1:20; to a second series 0.1 cc. of anti-B diluted 1:10. In the first tube of each series there is placed 0.1 cc. of saline; in the subsequent tubes, decreasing amounts of saliva (in a volume of 0.1 cc.). The tubes are well shaken and kept in the cold (7 to 10°) over night. Next day, to the first series A cells are added, and B cells to the second series (amounts as in the preliminary test). Agglutination is read after centrifuging. The following are typical results:

	Test tubes	
	Row 1 anti-A + A cells	Row 2 anti-B + B cells
Saline (control)	++++	++++
A saliva		
undiluted	−	+++
1:10	−	++++
1:100	−	++++
1:1000	±	++++
1:10,000	++	++++
B saliva		
undiluted	++++	−
1:10	++++	−
1:100	++++	−
1:1000	++++	−
1:10,000	++++	+

In this case isoagglutination was inhibited specifically by the saliva, even in rather high dilution. If the cells had been added sooner, say immediately after mixing the saliva and serum, inhibition would also have been obtained, but it would have been less marked.

2.754 The A-specific Sheep Hemolysin Test

This is applied in analogous fashion for the A characteristic. It is capable of detecting the group substances in saliva or sperm in dilutions as high as 1:10,000. That amount of immune serum is used which gives

complete hemolysis at 37° in 20 minutes. The following is an example of a test on a group A and a group B saliva:

2.7541 Titration of Sheep Hemolysin in an Anti-A Immune Serum

	Tube number				
	1	2	3	4	5
Immune serum (1:1000)	0.5	0.25	0.15	0.1	—
Saline	—	0.25	0.35	0.4	0.5
Sheep cells (5%)	0.25	0.25	0.25	0.25	0.25
Complement (fresh guinea pig serum, 1:10)	0.25	0.25	0.25	0.25	0.25
Reading after 20 min. at 37°	c	c	ac	str	0

c = complete hemolysis; ac = almost complete hemolysis; str = strong hemolysis; 0 = no hemolysis.

In the main experiment, 0.2 cc. of immune serum, diluted 1:1000, is used.

2.7542 Main Experiment. 0.2 cc. of sheep hemolysin diluted 1:1000, various amounts of saliva, plus saline to give 0.5 cc.; 0.25 cc. of 5% sheep cells.

Saliva		Hemolysis after 20 min. at 37° with	
Dilution	Amount	Saliva 1 (group A)	Saliva 2 (group B)
1:1000	0.5	0	c
	0.25	0	c
	0.15	0	c
	0.1	0	c
1:10,000	0.5	i	c
	0.25	str	c
	0.15	ac	c
	0.1	c	c
Saline	0.5	c	c

i = intermediate; other symbols as above.

2.76 Complement Fixation

A substrate which contains the group specific substance will, when it reacts with a strong specific serum in the presence of complement, cause the fixation of complement (96). The procedure is similar to the Wassermann reaction, the technic of which can be found in many sources, for instance in (20). The reaction is useful in experimental work, and in some circumstances is helpful in forensic investigations. It is applicable to blood group studies only when the group specific substances can be obtained in alcoholic solution. Therefore negative results should be interpreted with caution. Not every immune serum, even though it may con-

tain group specific agglutinins, is active in this way (1). For the detection of the B characteristic the so-called lipid-immune sera must be used. Rabbits are injected, not with washed erythrocytes, but with alcoholic extracts of corpuscles, mixed with protein (50, 97, 98). Also the best antigen to use in the complement-fixation experiment is an alcoholic extract of the material.

2.761 Procedure

To a diluted alcoholic extract of the material there is added group specific immune serum and complement. The mixture is allowed to stand 1 hour at 37° (fixation). Then sensitized sheep or bovine cells are added, and the tubes kept 20 minutes at 37° (second phase). The bovine system should be used in special experiments when it is desired to exclude the interference of the sheep lysin in the group specific immune sera. If complement was used up in the reaction of the first phase, there will be no hemolysis in the second phase.

2.762 Example

For the antigen we may use an extract of erythrocytes. One part of washed packed erythrocytes is mixed with 10 parts of 95% alcohol. The mixture is allowed to stand, with repeated shaking, for several days at room temperature. The fluid is then filtered off. Before use, 1 part of the extract is gradually diluted with 6 parts of saline, with continual shaking. This mixture is used in volumes of 0.25 cc. Complement, serum and saline are added to a volume of 0.75 cc.

The *hemolytic system* may be the same as that for the Wassermann test, i.e., sheep cells and titrated sheep lysin ("amboceptor") or bovine cells and their lysin. To each tube, after incubation, is added 0.25 cc. of the sensitized suspension.

Immune serum		Hemolysis after 20 min. at 37° with	
Dilution	Amounts	Extract of A cells	Extract of B cells
1:100	0.25	0	c
	0.15	0	c
	0.1	0	c
1:1000	0.5	0	c
	0.25	i	c
	0.15	str	c
	0.1	ac	c

Symbols as above.

2.77 Precipitin Reaction. (See above, sec. 2.3242).

This reaction is applicable only when water-clear solutions of the antigen and very strong sera are available. Because of its graphic nature, it is

well suited for purposes of demonstration, particularly to illustrate the existence of dissolved group specific antigens. Only certain immune anti-A sera can be used (sec. 2.3242).

2.771 Example

Clear group specific immune anti-A serum is placed undiluted in the bottom of small tubes (sec. 3.41312). Then human serum, diluted 1:50, from each of the four blood groups is carefully placed over this with the help of a capillary pipette (drawn out of glass tubing) and a rubber bulb. Almost immediately a "ring" is formed in the tubes containing the sera of groups A and AB, if they come from a person of the "secreting" type (figure 14).

2.78 Flocculation Reaction

Alcoholic extracts of erythrocytes of groups A and AB are specifically flocculated by suitable anti-A immune sera (49). Alcoholic extracts of organs may also be used. Specific flocculation with B extracts has not been reported.

2.781 Preparation of the Extracts

The washed sediment of one volume of blood is extracted (48, 49) with $2\frac{1}{2}$ volumes and again with one volume of alcohol overnight at room temperature, and the alcoholic extracts evaporated to one-half of the original blood volume. To 1 cc. of this fluid 5 cc. of saline are added drop by drop with constant shaking, a homogeneous stable emulsion being formed.

2.782 The Tests (49)

According to Landsteiner and van der Scheer, 1 part of alcoholic extract is blown from a pipette into 5 parts of saline. To 0.2 cc. of the inactivated rabbit immune sera, diluted one-half, 0.2 cc. of the emulsion is added, and the tubes kept at 37° for 20 hours. In the readings, both the formation of flakes and of sediment are taken into account. Reading is by means of a hand lens.

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SPECIAL APPLICATIONS OF BLOOD GROUPING TECHNICS

3. SPECIAL APPLICATIONS OF BLOOD GROUPING TECHNICS

3.1 Blood Transfusion. Transfusion Accidents

3.11 Choice of Donors for Transfusions

3.111 Principle

In spite of faultless transfusion technic, accidents will happen from time to time if the donor and recipient are not serologically compatible. Hemolysis of the introduced erythrocytes takes place almost instantaneously, and alarming clinical symptoms are immediately produced. Similar symptoms, although less severe, follow the injection of small amounts of incompatible blood, which formerly often occurred in the preliminary "biological test" (29).

Experience has shown that accidents may be avoided by taking account of the serological differences which find their expression in the blood groups, as originally indicated by Landsteiner (cf. 16). We may state as a general rule that any person who belongs to the same blood group as the recipient is suitable as a donor. Certain qualifications to this statement will be considered below.

When blood of a certain group is transfused into a patient of the same group, no danger is to be anticipated, since the group-specific agglutinins in the recipient's plasma will not agglutinate the donor's corpuscles, nor will the donor's agglutinins act on the recipient's cells. Theoretically, therefore, it should be possible to select a suitable donor simply by blood-grouping the patient and a number of prospective donors (selection of donors by blood group determination).

In practice, however, it has not proved to be sufficient simply to determine the four blood groups. Partly, no doubt, due to the presence of (usually weak) extra agglutinins and agglutinable factors in human blood, the bloods of prospective donor and recipient often have the power of reacting, even when of the same group. In such cases, use of the donor's blood may be followed by mild or even severe reactions.

The compatibility of the bloods of the donor and recipient may be directly determined by allowing the serum of each to act on the red cells of the other (*cross-matching*). If no agglutination is observed, the bloods are compatible, if they are of the same group.

It is sometimes asked which of these two tests (blood group determination or cross-matching) is to be preferred. The question, however, is incorrectly put. Each of the two tests has its special advantages and dis-

advantages, and a maximum of certainty is obtained only if the two tests are carried out together. The importance of a correct diagnosis is such that obviously nothing should be omitted which contributes to the certainty of the result.

3.112 Cross-matching

The advantage of cross-matching lies in the fact that it tests directly the thing which it is desired to know, namely, the relation between the bloods of donor and recipient. Individual antibody-antigen reactions, which might be independent of the group scheme, would be detected in this way. Further advantages are the simplicity of the test and its independence of any (sometimes unreliable) reagents.

At the same time, there is a danger. All controls are lacking, and if one of the components (cells or serum), or especially if both components at the same time, should happen to react rather weakly, then a positive reaction might be overlooked. Also, if the blood groups are not determined, there is the possibility that group antibodies which happen to be very weak in the serum (defective type) may nevertheless be present and active in the tissues (sessile antibodies).

3.113 Group Determination

This has the advantage that the test may be performed with known strong reagents, so that weakly developed agglutinogens or reactions of insensitive corpuscles may be detected. If the sera are tested, as well as the cells, the results serve as a useful check on the correctness of the group as determined from the corpucles.

Another advantage of group determination is that only those persons need be tested as prospective donors who are known to belong to the same blood group. Persons probably suitable may be selected from a previously prepared donor list.

In spite of the fact that it is probably hemolysis, rather than agglutination, which is so serious following transfusion of incompatible blood, it is customary to test only for agglutination, since this is far easier to do, and since iso-hemolysins do not seem to occur except in the presence of the analogous iso-hemagglutinins.

3.114 So-called "Universal Donors" and "Universal Recipients"

Transfusion of blood of one group into a patient of a different group is not always followed by undesirable reactions. In particular, the transfusion is often successful in cases where the introduced blood corpuscles do not meet with homologous antibodies in the serum of the recipient. The cases in which this holds true are shown in the accompanying scheme

(figure 21) where the arrows indicate the direction of possible transfusions from donor to recipient.

It may be seen that on this basis persons of group O may serve as donors to persons of any of the other three groups (and of course to persons of the same group), and persons of group AB may receive blood from persons of any of the three other groups, as well as from persons of their own group. For this reason persons of group O have been called "universal donors", and persons of group AB "universal recipients".

These designations, especially the former, have caught strong hold of the popular imagination, and many a layman will proudly state that he is a "universal donor". The terms are nevertheless objectionable, for

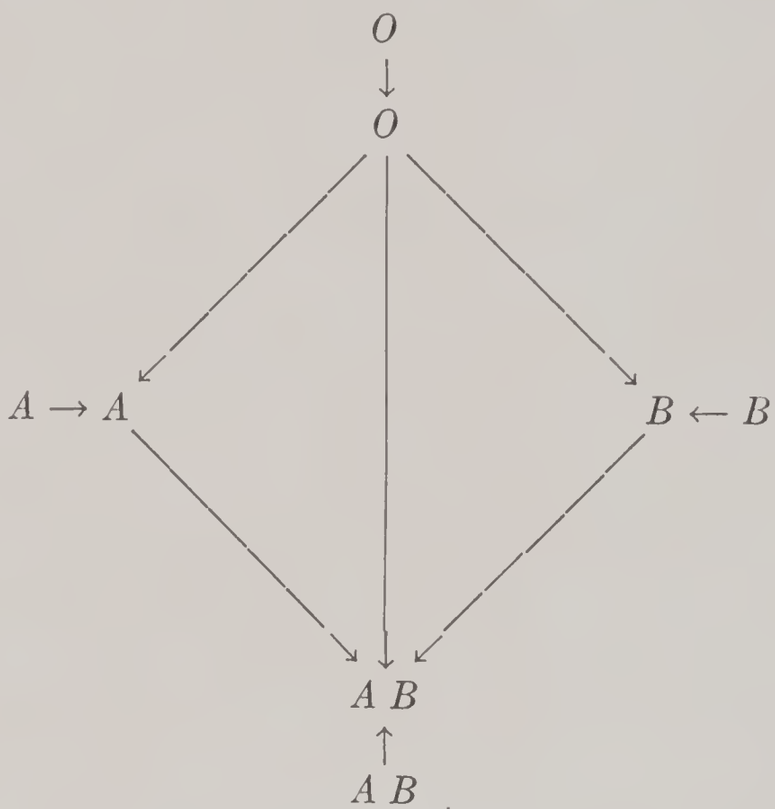


Fig. 21. Various possibilities of transfusion

they tend to create the impression that transfusions from universal donors or to universal recipients are always without danger. This is not true; serious reactions have occasionally been observed following such transfusions (42).

Universal donors are not to be used indiscriminately as a routine procedure, but only when a suitable donor of the proper group is not available. In such cases the possible risk involved should be clearly borne in mind from the beginning. Probably safe are universal donors whose serum contains only a low titer of anti-A and anti-B agglutinins, especially if their blood lacks the factor Rh (41). If it is wished to use such donors, a number of persons of group O should be examined for their agglutinin titer, and a suitable record made.

3.115 Repeated Transfusions

On repetition of a transfusion the preliminary serological tests must each time be carried out anew, even in case a donor already tested is being used again, for there is always the possibility that the previous transfusion may have induced the production of *immune* iso-antibodies against individual group substances of the donor. In particular, there have been a number of cases suggesting that anti-Rh agglutinins are particularly likely to cause trouble (42, 20, 41).

Thus it is necessary to test each time whether the serum of the recipient will agglutinate the corpuscles of the prospective donor. This is accomplished by simple cross-matching. It is sufficient to test the donors' cells with the recipient's serum, (Table 21 ,tube 9). Since anti-Rh agglutinins are often active only at 37°, a duplicate tube should be incubated at this temperature for 10 to 15 minutes, then centrifuged slowly (21, 41).

3.116 Requirements for the Technic

The physician who carries out blood transfusions demands two things of the serological technic:

(1) The technic must be unconditionally 100% reliable.

(2) The method must give the result *quickly*, if possible in a few minutes.

The first demand can be waived in extreme circumstances only. As a rule, everything else must be subordinated to reliability, for an erroneous determination may be followed in a very short time by a fatal accident.

From the results of a questionaire to which 350 hospitals responded, Levine and Katzin (19) conclude that in 1935 and 1936 these hospitals experienced at least 60 accidents following transfusion, of which 16 were fatal. It was reported that in 36 cases incompatible blood had been administered.

The second requirement, rapidity, is basically justifiable also. It seems to be carried too far in some quarters, however. It must never be allowed to lessen the stringency of the first requirement.

Emergency cases do come up, of course. We may distinguish two sorts: first, a small number of very urgent cases, and second, a much larger number of cases where at least 15 to 30 minutes are available for the preliminaries. Under modern conditions, the danger of erroneous group determinations is almost entirely confined to the first group. In a properly organized institution the extremely urgent cases constitute only a small per cent of the total, so it would be foolish to base the general routine technic on the procedure which has to be followed in the exceptional cases. We shall therefore first discuss a technic which may be followed in routine cases, which make up by far the majority.

3.117 The "Complete Test"

For *maximal* certainty, when a few cubic centimeters of blood are available from both donor and recipient, two tests, blood group determination and cross-matching, are set up independently.

3.1171 Group Determination:

a. Group determination of donor and recipient with test sera A (anti-B) and B (anti-A).
b. Group determination of donor and recipient with test corpuscles A and B.

3.1172 Cross-matching:

a. The recipient's serum with the donor's corpuscles.
b. The donor's serum with the recipient's corpuscles.

3.1173 Procedure

A corpuscle suspension is prepared from the donor and from the recipient by mixing a few drops of blood with diluting fluid. If the blood has clotted and unusual difficulties are experienced in pouring off corpuscles from the clot, a glass rod or a pipette may be stuck into the clot and washed off into the salt solution until the desired density of suspension is obtained.

Ten test tubes are needed for the test, four for determination of the blood group of the donor, four for the recipient, and two for cross-matching.

Since the corpuscles to be investigated may always be tested immediately after being taken, while at least a few minutes must elapse before serum separates, the first four tubes are used for the corpuscles and the serum to be tested is placed in the next four. In the last two tubes (9 and 10), are mixed the corpuscles and serum of donor and recipient. Details are shown in the adjoining scheme:

Scheme of the "complete test" for choice of a donor

Tubes 1–8 *group determination*

1–4 for *corpuscles*

1, 2 of the donor
3, 4 of the recipient

5–8 for the *sera*

5, 6 of the donor
7, 8 of the recipient

Tubes 9, 10 *cross-matching*

9 donor's cells and recipient's serum
10 recipient's cells and donor's serum

The details of the procedure may be seen from table 21.

If at the beginning of the experiment cells and serum are already available, then the whole experiment is set up at once. The test tubes are filled according to the scheme, and the contents well mixed by shaking, then they are strongly centrifuged for two minutes. When the tubes are removed from the centrifuge, the whole experiment may be read (cf. sec. 2.251) and recorded.

If the blood is received immediately after being taken, as in urgent cases, serum will not yet be available, but the cell suspensions may be prepared immediately. These are placed in tubes 1 to 4, and (to be used later) in tubes 9 and 10; the corresponding test sera are added to the first 4 tubes and these tubes shaken and centrifuged. At the same time,

TABLE 21

"COMPLETE TEST" FOR CHOICE OF A BLOOD DONOR

(Amounts of reagents given in cc.)

Tube no.	Corpuscles of		Serum of		Known (test) blood			
					Serum		Corpuscles	
	Donor	Recipient	Donor	Recipient	A (anti-B)	B(anti-A)	A	B
1	0.2				0.1			
2	0.2					0.1		
3		0.2			0.1			
4		0.2				0.1		
[5			0.1				0.2	
[6			0.1					0.2
[7				0.1			0.2	
[8				0.1				0.2
9	0.2			0.1				
[10		0.2	0.1					

The symbol [indicates tests which are commonly omitted in routine laboratories (sec. 3.118).

blood from the donor and the recipient is centrifuged to obtain serum (or defibrinated blood prepared).

After two minutes tubes 1 to 4 may be read (figure 10) and a preliminary decision made, particularly if the donor is to be rejected because of incompatible group. Then the sera should be examined for agglutinins. If sufficient serum has separated from the bloods of the donor and the recipient, 0.1 cc. of the donor's serum is added to tubes 5, 6, and 10, and 0.1 cc. of the recipient's serum to tubes 7, 8, and 9. If sufficient serum has not separated, then the tubes containing their bloods are centrifuged again until serum separates. It is then added, as above, to tubes 5 to 10. In tubes 5 and 7 are placed test corpuscles A and in 6 and 8 corpuscles B. After shaking, they are centrifuged and read.

The whole procedure lasts 3 to 5, at the most 10, minutes, if serum separates promptly from the blood of the donor and the recipient. If it does not, another 5 to 15 minutes may be needed. The procedure is made more rapid if the test has been planned before the blood arrives, and tubes 1 to 4 have already been filled with test serum, and tubes 5 to 8 with test cells.

The tubes may be labeled with a wax glass-marking pencil. In some laboratories it is considered convenient to have test tubes with different colored permanent labels or colored rings near the top, to make identification easier. For instance, blue-ringed tubes may be used for test blood A, red-ringed tubes for test blood B.

The names of patient and donor, or the number of the experiment as entered in a notebook, should be written on a tube or on a label, or on a slip of paper and affixed to the rack carrying the tubes.

3.1174 Controls. The choice of suitable testing sera is discussed in sec. 2.221 (cf. 38). They should have been tested against suitable bloods recently enough to ensure their activity. It is clearly undesirable to wait until an urgent transfusion case comes in to do this. The test corpuscles should be from individuals whose cells are known to be normally reactive.

Controls can be included along with the blood group determination, in which case table 21 is completed by addition of the following tubes:

	Expected result
I. Control of the test sera	
1. Test serum A (anti-B)* + test cells A	−
2. Test serum A (anti-B)* + test cells B	+
3. Test serum B (anti-A)* + test cells A	+
4. Test serum B (anti-A)* + test cells B	−
II. Control of the test cells	
1. Test cells A* + test serum A (anti-B)	−
2. Test cells A* + test serum B (anti-A)	+
3. Test cells B* + test serum A (anti-B)	+
4. Test cells B* + test serum B (anti-A)	−

(The material being controlled is designated by *)

If the expected results are obtained in this test, then the test corpuscles belong to the correct type. At the same time it should be noted whether the reactions are sufficiently strong.

The sera can also be tested occasionally in dilutions of, say, 1:5 and 1:10. A good serum should give agglutination when diluted 1:10.

Any test serum or cells giving results contrary to expectation in the control test should of course be discarded.

In each test the findings are immediately entered into a special note-

book, or a previously prepared protocol sheet. This is necessary, not only to obtain a view of the results of the experiment as a whole, and to avoid errors, but also so that if questions should arise later (transfusion accidents), all the evidence will be at hand. In the protocol must also be shown the source and identity of the reagents (test sera and cells). (See table 22).

If there is no special hurry, for example, when it is planned to perform the transfusion the following morning, it is not necessary to centrifuge the tubes. They may be simply allowed to stand with occasional shaking and read after an hour or so at room temperature. This is particularly convenient when a large number of bloods are being tested simultaneously,

TABLE 22

PROTOCOL OF AGGLUTINATION TESTS FOR CHOICE OF BLOOD DONOR FOR JOHN SMITH

No.	Name	First name	Donor (D) or recipient (R)	Corpuscles tested with known serum		Serum tested with known cells		Cross-matching		Blood group	Suitable donor
				B (anti-A) (no. 26)	A (anti-B) (no. 80)	A (no. 90)	B (no. 89)	D cells + R serum	R cells + D serum		
1	Smith	John	R	−	−	+	+			O (anti-A, anti-B)	
2	Smith	Mary	D	−	+	+	−	+	−	B (anti-A)	
3	Smith	George	D	−	−	+	+	−	−	O (anti-A, anti-B)	√
4	Burns	Frank	D	−	−	+	+	−	−	O (anti-A, anti-B)	√

√ indicates that the individual is a suitable donor for this patient. Names are fictitious.

as in preparing a donor list. Levine (18) advises centrifuging only the cross-matching test (he does the grouping on slides) at 500 RPM for 1 minute.

It is desirable to have on hand absorbed immune anti-A and anti-B sera (see above, sec. 2.3) with which to retest the patient's cells in case of doubtful reactions. Such immune sera can be made stronger than any ordinary human sera, and in addition provide a valuable control on the specificity of the latter.

3.118 Permissible Changes in the Technic

We have described the technic which appears to us to give the greatest security against erroneous grouping, and possible resulting transfusion accidents. In addition to its reliability, this technic is very rapid. Never-

theless there may be workers in some institutions who will have enough confidence in their technic, particularly after long experience, to attempt to simplify somewhat the methods presented above. In addition, there will be instances, such as in bedside cases, where proper assistance and apparatus will not be available to carry out the test as outlined, and some simpler technic will be imperative. We therefore proceed to describe other methods, but cannot assume responsibility for any errors resulting from their use. Probably when used by persons familiar with them, and with due attention to controls of the specificity and potency of the typing sera, they will be on the whole reliable.

3.1181 Omission of Test of Patient's Serum. It is recommended that the patient's serum be always tested, for by so doing mistakes in grouping are much less likely. Also, there is a better chance of detecting irregular agglutinins which may cause reactions, for example, when a person of group A is transfused with blood from a donor also in group A, but belonging to a different sub-group (7). Nevertheless Wiener (40) points out that a survey of American hospitals reveals that, with few exceptions, only the cells and not the serum are tested, even in New York City, although the New York City Sanitary Code for 1930 requires that both tests be done. Directors of hospital laboratories may decide for themselves whether the record of the hospitals questioned by Levine and Katzin (see above, sec. 3.116) is encouraging enough to incline them to imitate these institutions in routinely omitting the test of the serum. If the serum is not tested, we recommend the inclusion of controls in the form of cells of known groups, set up with the first tests of each day, or each time a new batch of test serum is used. Levine (18) does not consider cross-matching the donor's serum and the patient's cells (tube 10, table 21) essential.

3.1182 Very Urgent Cases. In emergencies one may be contented with group determination of the corpuscles of donor and recipient (tubes 1 to 4 of the above scheme). If the donor belongs to group O, it is even possible to omit the test of the recipient's blood (tubes 3 and 4). But in such cases, the determination of tubes 1 to 4, or 1 and 2, should be carried out in duplicate with two different kinds of test sera A (anti-B) and B (anti-A), say with human and immune test sera, as suggested above.

3.1183 Tests on Slides or Tiles. If the usual serological apparatus (pipette, test tubes, centrifuge) is not available, the reaction may be carried out on microscope slides. This method is also desirable when test serum is available only in very small quantities. The procedure for group determination then takes the following form:

A drop of the blood to be tested is added to physiological saline and evenly mixed by shaking or stirring; this is the cell suspension. On the

left of the slide is placed a large drop of the test serum A (anti-B), and on the right an equal drop of test serum B (anti-A). The position of the sera may be indicated on the slide with a wax glass-marking pencil. A large drop of cell suspension is added to each drop of serum. To prevent pseudoagglutination it may be desirable to dilute the test-sera 1:2 before using them in the slide technic. Complete mixing is secured first by stirring with a platinum loop or a clean glass rod, later by rocking the slide to and fro. The progress of the reaction is observed against a white background. With strong serum, the test is not observed for more than 5, or at the most 10, minutes. The diagnosis scheme is shown in figure 22.

Both the patient's and donor's cells should be tested in this way, and on a third slide the patient's plasma or serum is tested against standard cell suspensions. The cross-matching tests (on the left, patient's serum and donor's cells, on the right patient's cells and donor's serum) are made on a fourth slide. At the end of 5 minutes, each mixture may be covered with a coverslip and the reactions read under the low power of the microscope. Blood grouping tests are probably carried out on slides in most of the hospitals of the United States (cf. 38).

In place of a slide, a glass plate painted white on the bottom, or a porcelain plate with depressions, or the white opal glass tile recommended by Riddell (32) may be used. Riddell's technic of adding whole blood to the serum has already been described in sec. 2.2522.

3.1184 Test Reagents Unavailable. If no test blood, particularly no test serum, is available, then a cross-matching test (tubes 9 and 10) must suffice. A positive agglutination in tube 9 or 10 is sufficient definitely to exclude a prospective donor. If the result of the cross-matching with the donor's cells (tube 9) is satisfactory, in very urgent cases involving life and death, the transfusion may be begun without waiting for the results of further tests. It will be realized that this is an extreme measure.

3.1185 Lattes' Slide Method. If separation of the serum is delayed, and a quick decision is imperative, then in place of tubes 5 to 10 (and in some cases with the omission of tubes 5 to 8 and 10), the whole blood instead of serum may be tested, according to the method of Lattes (17). A small drop of the recipient's blood is placed on a slide and spread out so that it dries rapidly with a diameter of a few millimeters. A drop of cell suspension from the donor (1 to 2 drops of blood in 1 cc. of saline) is placed on the slide near the dry blood. Then a cover-slip is placed on the slide so that the moist drop spreads to the edge of the dried blood and makes contact with it. Separation of the drops by air bubbles is to be avoided.

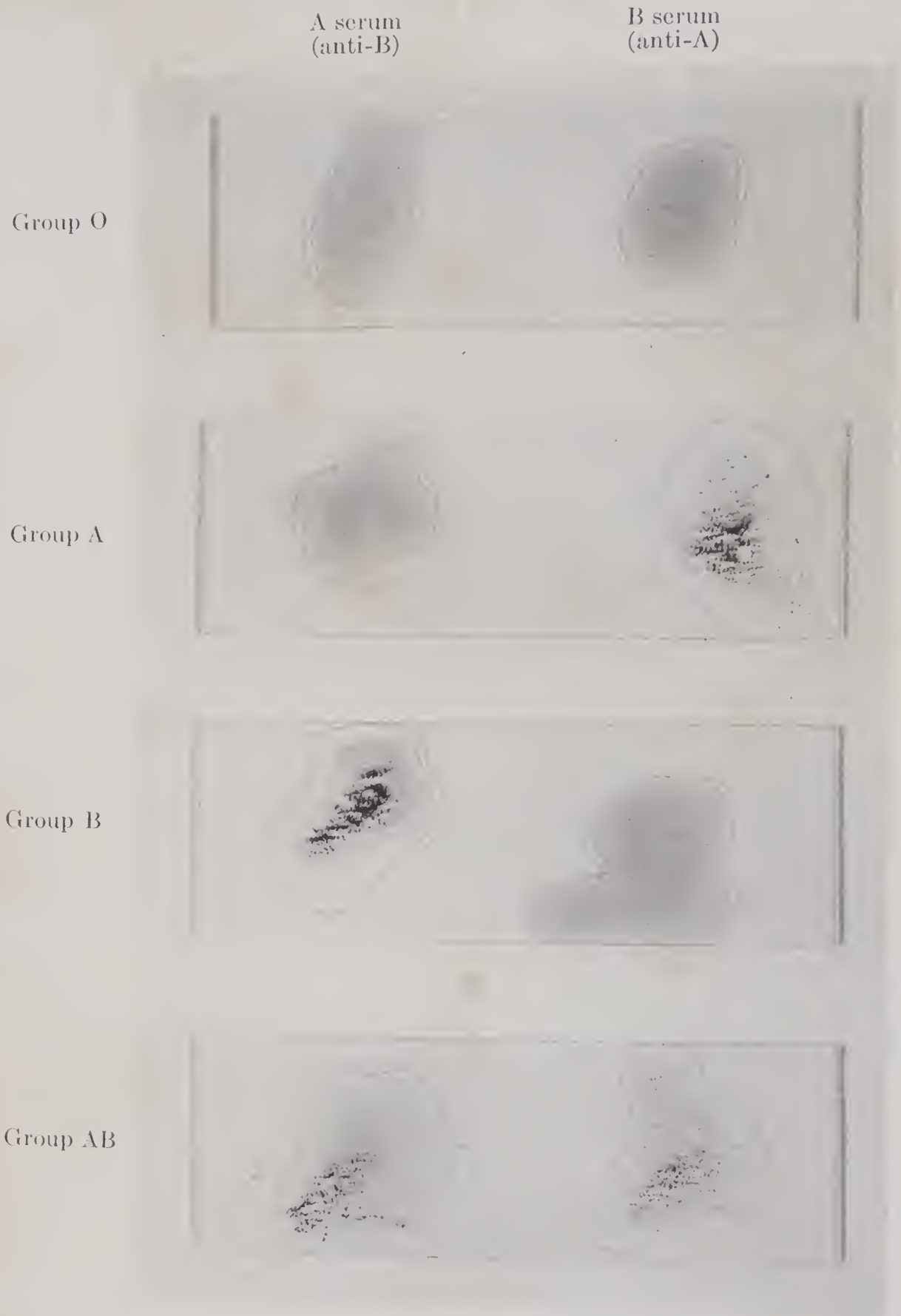
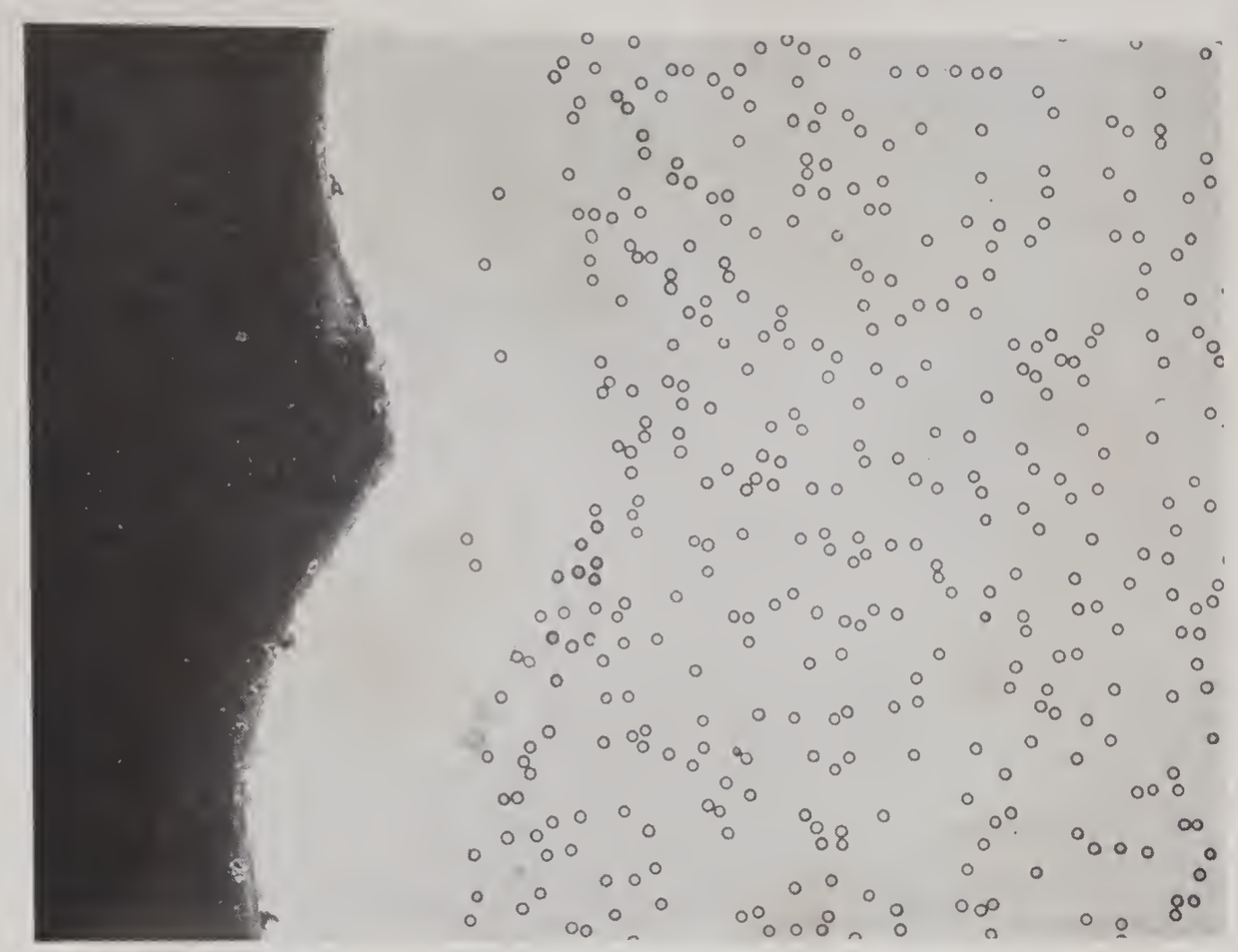
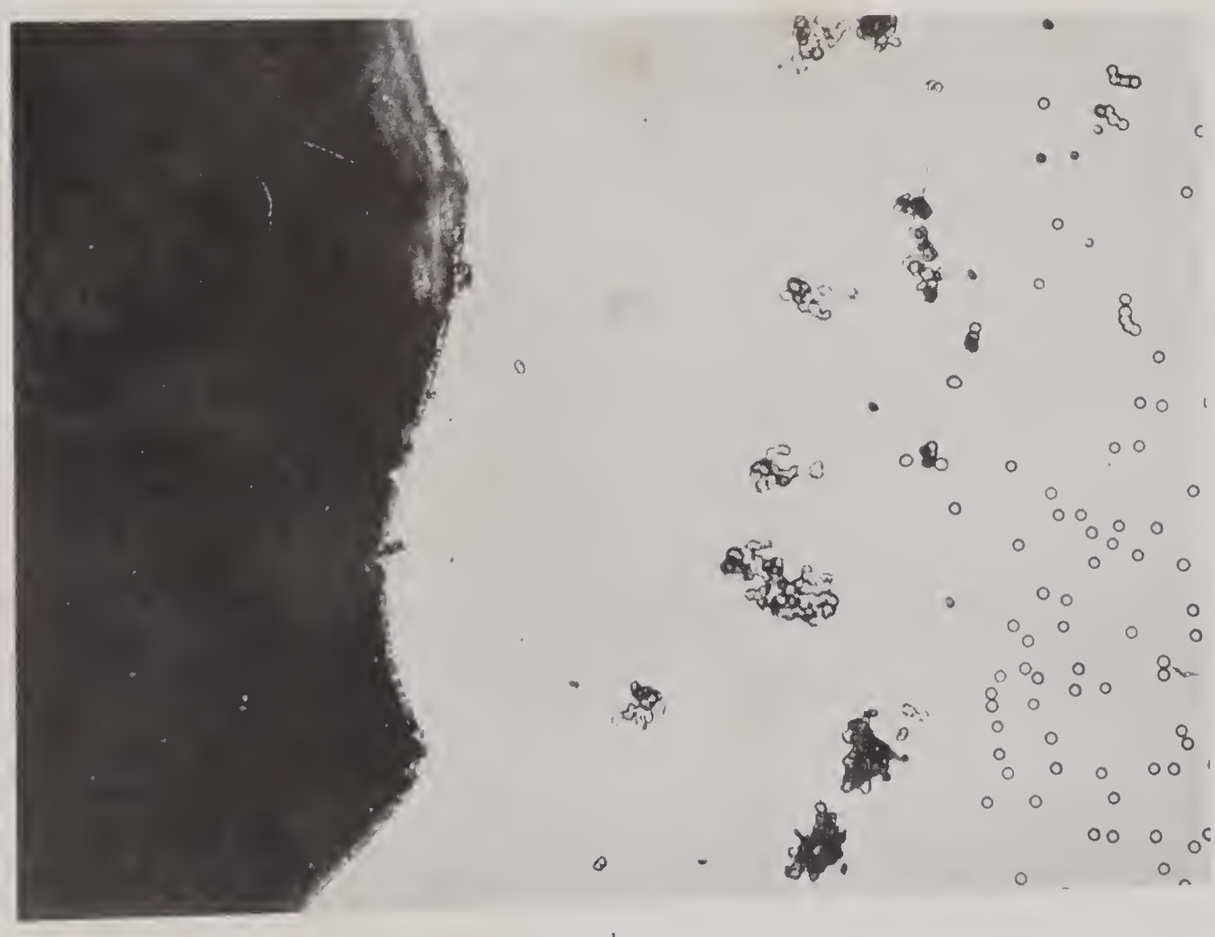


Fig. 22. Blood grouping on slides (actual size)

In a positive reaction, typical agglutinated clumps are formed, after a few minutes, in the donor's cells (figure 23). In a negative test, the donor's cells in the zone of mixture are as evenly dispersed as in regions more distant from the dried blood of the patient.



a



b

Fig. 23. Lattes' slide test with dried blood: (a), negative reaction; (b), positive reaction.

Since concentrated serum is being employed here, if positive agglutination results, a check on possible pseudo-agglutination must be made. This is done most simply by removing the cover-slip and replacing it. If only pseudoagglutination is present the clumps break up, but if the agglutination is specific, they remain, or even extend through the whole preparation.

Lattes' test is rapid and easy to carry out, but requires more practice to read than the test tube reaction.

3.1186 Errors. Mistakes can certainly be avoided by following the complete technic as given above; otherwise some errors may be made. Errors to which selection of a donor may be subject are discussed in the following references: (39, 19, 40, 26).

3.12 The Transfusion

Since this book deals solely with blood grouping technic, it is not proposed to discuss the technic of transfusion. Sufficient details may be found in the following references: (39, 32, 4).

We may offer this final advice: no matter how confident the surgeon may be of the correctness of the laboratory's choice of a suitable donor, the patient should be carefully observed during the first minutes of a transfusion, so that this period becomes, in effect, a "simple preliminary biological test" of compatibility.

3.13 Further Examination of Donors

In addition to the possession of compatible blood, there are a number of qualifications which a donor must have to be suitable. In order to avoid the transmission of disease from donor to recipient, and thus possibly doing more harm than good by the transfusion, it is necessary to make sure that the donor is in good health. It is unfortunate that examination of the donor, supplementing the blood grouping tests, is so often superficial or practically non-existent. As a result, in quite a number of cases syphilis has been transmitted from donor to patient.

Examination of the donor takes two forms: a physical examination, and the laboratory tests.

3.131 Physical Examination

The physician should satisfy himself that the donor is free from communicable disease. The exact points to be covered will vary with the individual and the institution. For a very strict standard, see the appendix to this section, which gives the regulations of the Department of Health of New York City.

Syphilis should be particularly looked for, and when the history is taken, adequate inquiry should be made regarding malaria, for this disease can be transmitted even from donors who contracted it decades before the

date of transfusion. Any suggestion of existing hemolytic streptococcus infection, such as a septic sore throat, should be regarded very seriously. Transmission of gonococcal infection or tuberculosis has not yet been recorded, but nevertheless donors with either of these conditions in active form are to be rejected.

Also, the condition of the donor should be considered from the point of view of his own welfare. Donors must not be used who will be harmed by the transfusion; therefore volunteers with anemia, heart disease, hyperthyroidism, etc., will be rejected.

3.132 Laboratory Examination

The same sample of blood which is withdrawn for grouping and cross-matching will also be sufficient for the additional laboratory examinations of the donor's blood which are desirable.

3.1321 Hemoglobin. The hemoglobin should be measured. The minimum requirement is 85%.

3.1322 Test for Syphilis. One or more serological tests for syphilis should be carried out. If plenty of time is available, any of the common tests may be made. If time is a factor, as it often is, one of the flocculation tests, such as the Kahn, Kline, or Hinton test, which can be done in a short time, should be carried out. In most of these tests a positive reaction is not definite proof that the individual has syphilis, but of course any donor giving a positive test will be ruled out, to be on the safe side.

On the other hand, the use only of donors giving a negative flocculation test will not afford an absolute guarantee that syphilis will never be transmitted by a transfusion, but such transmission will certainly be extremely rare.

3.1323 Other Diseases. It is doubtful if any laboratory tests will ensure against transmission of the other communicable diseases. Here the entire reliance must be on clinical examination. Malaria, for instance, has been transmitted in cases where the blood picture of the donor was negative. Laboratory tests are supposed always simply to supplement a thorough physical examination (25).

3.14 Obtaining a Donor

3.141 Relatives and Friends

The use of these volunteers has the advantage that they are usually willing to give blood, and that there is no cost to the patient. There is the disadvantage that nothing exact is known of their health or blood group, and unless there is time and opportunity for a laboratory and physical examination, there is danger of using an unsuitable donor. If it becomes more common, as seems likely, for all persons to have their blood

groups determined, and to carry some record of it with them, one of these disadvantages will disappear.

3.142 Organized Donors

When friends and relatives cannot be used, donors are obtained through hospitals or private donor agencies. In this country these donors are mostly "professionals", that is, persons who are paid for their services, and who give blood periodically whenever there is demand for it. In some countries volunteer donors have been extensively organized. This has great advantages, especially for poor patients.

3.1421 Professional Donors. In the United States the professional donor is the major source of blood for transfusion therapy (12). The cost of 500 cc. of blood varies from 25 to 50 dollars. This is prohibitive to the families of very poor patients. One large municipal hospital in 1939 paid 14,825 dollars to professional donors for urgent transfusions.

The donor agencies have lists of donors, arranged according to blood group. They make a medical examination and can almost invariably be relied on to furnish donors of the correct group. In New York City, donors and donor agencies are under the supervision of the Department of Health, and the whole organization is subject to strict regulations. These are reproduced *verbatim* in the appendix to this section.

Professional blood donors can be registered only after their serological test for syphilis has been found negative. Each professional donor must have his blood group established by suitable tests performed at laboratories possessing a permit from the Department of Health. Detailed directions are given for the physical examination to be given all professional and voluntary donors immediately prior to transfusion.

A good agency has available donors of all blood groups, including the rarer types, and their grouping can be relied on. Some agencies also take care of a medical examination immediately before sending out the donor. This will facilitate matters for the physician who performs the transfusion, but will not release him from his personal responsibility to make sure that he employs only suitable donors.

An example of the type of form used by a good agency is shown in figure 24.

3.1422 Volunteer Donors. It has been found in some cities that there are enough persons willing to give their blood for transfusions without financial return, so that it is not necessary to pay for blood if the volunteers are properly organized, and made to feel that in giving blood they are performing a service to society. This type of organization was carried out enthusiastically in London and Rotterdam. The British Red Cross Society in 1921 established the voluntary system, in which the donor receives no

(This form to be filed with patient's chart)

DOCTORS' DONOR SERVICE
1054 EAST 38TH STREET
BROOKLYN, NEW YORK
TELEPHONE ESPLANADE 7-2200-1

RECORD OF BLOOD TRANSFUSION

Patient — Hospital No
Ward — Bed — Room — Date
Service Dr.
Diagnosis
Indication
Previous transfusions, if any

DONOR — AGE — REG.NO. — GROUP — INTERNTL. JANSKY MOSS

Date:
Time:

PHYSICAL EXAMINATION:

Blood Pressure — Hemoglobin %; Gms/100cc ; Method
Pulse — Temperature
Condition of Genitalia — History or evidence of

- Heart
- Mouth
- Lungs
- Pharynx
- Skin
- Liver
- Spleen
- Lymphatic Glands

- Hyperthyroidism
- Drug Addiction
- Asthma
- Syphilis
- Communicable Disease

Other notable findings

Place of examination — Examining Physician

SEROLOGICAL EXAMINATION:

Reactions were not obtained in serological tests for evidence of syphilis

Microprecipitation tests performed — Technic.
Complement-fixation test performed — Technic.
Serologist

Operator
Reaction to Transfusion — Crossmatch
Result of Transfusion — cc Transfused
If reaction, probable cause
If Transfusion not given, contra-indication
Remarks:

Fig. 24. Typical form used by donor service (*courtesy of Doctors' Donor Service*)

payment except for travelling expenses, usually amounting to the equivalent of 25 cents.

Riddell (32) devotes two chapters to a thorough description of the merits and organization of this remarkable service. He emphasizes the low cost—about $2.25 per transfusion, for administration costs—, the high type of donor obtained, the freedom of the donors from syphilis (a professional who receives money for transfusions is less likely on the whole to refrain voluntarily from giving blood after acquiring a venereal infection), and the methods of supervision of the donor's health, which limit the number of times a year that blood may be given to four for men and three for women.

The voluntary system has proved practical and economical to administer, and permits the unlimited use of transfusions for indigent patients, without undue burden on the patient or the hospital. The administrative costs have been defrayed by income derived mainly from fees from private cases, with a smaller contribution by public institutions.

In London, in 1938, there were 2,698 donors on the list, and 6,628 calls for transfusion were received. There were at that time 46 affiliated services for the rest of England. When war broke out in 1939, a number of the London hospitals issued appeals for volunteers to give transfusions which would be needed for casualties from bombing.

In smaller towns difficulty may be experienced in finding donors, and there is a considerable field for individual endeavor there. Most hospitals of any size, however, have at least a few persons of known group available who can be called on.

3.15 Preservation of Blood. Blood Banks

3.151 History

The idea of storing blood for use in transfusion is not new, having been suggested during the first World War by Rous and Turner (34). Robertson (33), then of the U. S. Army, discussed the possible use of preserved blood from the depots we now call "blood banks".

Little was done about these suggestions in the next 20 years, and the present interest in the use of preserved blood was initiated by the reports of a number of Russian workers. Yudin (46) of the Sklifasovsky Hospital in Moscow told one of us in 1935 that he was the first to make use of preserved blood in this way, basing his work on experiments of Shamov on dogs; at the same time he demonstrated his technic of storing blood.

Yudin's work was done with blood from cadavers, and his method of preservation was very simple; the blood was mixed as it was extracted with 4 per cent sodium citrate solution, then stored at a temperature of 1 to 2°C. The workers at the Moscow Institute of Blood Transfusion proposed a more complicated preservative solution, and this was imitated,

more or less accurately, by a number of workers in this country, following the lead of Goodall *et al.* (11). The opinion of the majority of workers apparently now inclines to reversion to the original glucose-citrate mixtures of Rous and Turner and Robinson, or simple modifications of them. The addition of glucose to the citrate seems definitely to increase the preservative action.

TABLE 23

SOLUTIONS USED BY VARIOUS WORKERS IN STORING BLOOD (MODIFIED FROM (28))

Authority	Constituents	Concentration (per cent)	Proportionate amounts used	
			Volumes of solution	Volumes of blood
Saxton	Sodium citrate	3.8	10	100
Yudin	Sodium citrate	4.0	?	?
Bagdosarov	Sodium citrate	6.0	10	100
Robertson	Sodium citrate	3.8		
	Glucose	5.4		
Page et al.	Sodium citrate	1.25		
	Sodium chloride	0.85	10	10
Domaning	Sodium citrate	1.0		
	Glucose	2.0		
	Sodium chlorate	0.02	10	10
Duran Jorda	Sodium citrate	4.0		
	Glucose	1.0	10	90
DeGowin et al.	Sodium citrate	3.2	2	
	Glucose	5.4	13	10
Muether and Andrews	Sodium citrate	0.43		
	Glucose	4.68		
	Sodium phosphate (monobasic)	0.025		
	Sodium phosphate (dibasic)	0.25	15	10

A survey of some of the preservative solutions which have been proposed is shown in table 23, modified from (28).

The limitations to the use of stored blood vary with its state of preservation (8, 15, 43).

3.152 Changes in Preserved Blood

It has been demonstrated that a number of changes occur, varying considerably with the type of preservative mixed with the blood. The eryth-

rocytes gradually swell, increase in fragility, and eventually hemolyze (12, 43). The length of time that transfused cells remain in the recipient's circulation is decreased. Wiener and Schaeffer (43) and Levine (18) found that, while fresh blood or blood only a few days old survived in the circulation up to 3 or 4 months, blood stored for longer periods disappeared from the patient's circulation sooner; blood stored for as long as 21 days survived only about 24 hours. The appearance of icterus could not be detected in patients receiving blood less than 8 days old, while icterus regularly occurred, at times together with hemoglobinemia and hemoglobinuria, in patients receiving older blood.*

The platelets and white cells decrease within 24 hours (24, 15), the bactericidal and phagocytic activities are slowly reduced, the prothrombin concentration gradually diminishes, certain chemical changes are progressive. It has been demonstrated (35) that a marked shift of potassium ions from the red cells to the plasma quickly occurs, and for this reason Scudder *et al.* advise against the use of blood more than 5 days old. Wiener and Schaeffer point out that such blood might constitute a particular danger for patients with nephritis.

3.153 Period of Storage

The maximum period of storage of blood which is allowable is debated in the literature, with most observers suggesting that the blood be used within a week. Wiener and Schaeffer suggest that the limit should certainly not be more than 10 days.

3.154 Advantages

The advantages of being able to use stored blood are practically self-evident. If an adequate "bank" of blood is available, blood of all groups is constantly available, and the difficulty and delay often experienced in finding a donor are eliminated. It is possible almost to dispense with professional donors, to the advantage of the institution and the patient. Blood (not necessarily of the same group as that used) may be taken from a volunteer from among the friends and relatives of the patient. Since transfusions of blood of any particular group will, in the long run, be needed in direct proportion to the frequency of that group in the population, the stock of all groups can be kept adequate by taking blood at random from all suitable volunteers.

Since plenty of time is available for examination, the blood grouping and serological tests for syphilis on blood for "banks" should be on the whole more carefully and reliably done. This should practically eliminate the use of blood of the wrong group, although it can not be expected that any

* Levine in unpublished experiments obtained similar results (18).

organization administered by human beings will ever be entirely free of errors. Indeed, at least one classical hemolytic reaction following transfusion of stored blood has already been reported (3), due to forwarding the wrong bottle of blood to the ward.

The advantages of stored blood in wartime are particularly great, and the first suggestion for its use was in this connection. The difficulties of having the right donor at the right time are greater than in civil life, and the urgency of the transfusion often greater. Much can probably be accomplished by systematically grouping all soldiers, and arranging that all members of a given military unit be of the same blood group, as rumour has it is being done in one of the armies engaged in the present conflict, but the use of stored blood and plasma (see below) will probably always provide important advantages.

3.155 Organization and Conduct of Blood Banks

The organization and the arrangement of the plant will probably differ in each individual case, and it would hardly be worth while to discuss the matter here. A photograph of the blood bank at the Massachusetts Memorial Hospitals is shown in figure 25.

The technic of setting up a blood bank and collecting and storing blood has been fully described in (8, 47, 9, 6, 32, 31, 5, 2).

3.156 Sources of Blood for Storage

3.1561 Donors. The obvious and probably the best source of blood for storage, volunteer donors, has already been mentioned; there are certain other sources which may also be mentioned.

3.1562 Corpses. Yudin introduced the use of blood from persons who had died from causes other than communicable disease (46); this source has historical interest as the first source of blood for storage. He demonstrated that the blood of cadavers, which is still unclotted several hours after death, could be extracted and stored. Such blood, provided group determination, test for sterility, and serological tests for syphilis had been done and a thorough autopsy had excluded the possibility of infection, was found to be satisfactory for use in transfusion.

It is unlikely that this source of blood will become popular in this country in the near future, unless it should become much more difficult than it is at present to obtain live donors.

3.1563 Placental Blood. Goodall *et al.* proposed the use of the 100 cc. or so of blood which is contained in the placenta and which is ordinarily lost after delivery (11). This source of blood for blood banks has also been advocated by Barton and Hearne (2) and others. Such blood seems

suitable for use in transfusion (30). Its use has certain disadvantages, however; it is more difficult to obtain sterile, only 50 to 100 cc. is obtained from each case, of which a perceptible fraction is used up in groupings and tests for syphilis.

Fig. 25. Blood bank at the Massachusetts Memorial Hospitals; General Electric cabinet No. 2425 (*courtesy of Dr. F. E. Barton, Visiting Surgeon and Director of Transfusion Service*).

3.16 Substitutes for Blood

In many cases, when transfusion is indicated there is nothing which will take the place of blood. In others, when it is wished simply to restore the volume of the circulation, substitutes may be considered.

3.161 Saline

The use of saline to restore the blood volume is not new; we shall not discuss it here, for it is not satisfactory except as a very temporary expedient, since it is soon lost by diffusion, owing to the low molecular weight of sodium chloride.

3.162 Gum Acacia

Solutions of gum acacia are not readily diffusible, and for this reason such sterile solutions were introduced in the first world war to restore blood volume after hemorrhage. The gum seems to persist in circulation for a long time, so the measure was in some degree successful. The practice has since been nearly abandoned, however; just why is not clear. Some have claimed that liver damage eventually resulted in many patients.* Amberson (1) considers acacia a useful substitute.

3.163 Pectin

Hartman *et al.* (14) have proposed the use of pectin solution ($\frac{1}{2}\%$) as a blood substitute in shock. Pectin has a high molecular weight, is apparently non-antigenic, and non-toxic. The source, method of manufacture, and ease of preparing and sterilizing stable buffered solutions make pectin readily available. The material is mostly eliminated, through the urine, in 48 to 72 hours. Only 8 clinical cases of its use have been reported, however.

3.164 Serum and Plasma

3.1641 Principle. Since the patient can stand a drastic reduction in hemoglobin, it is clear that for some purposes plasma or serum is the ideal substitute for blood. They have been used to some extent for other purposes for many years (1, 37). Plasma can be used after long storage, since removal of the erythrocytes ends the possibility of hemolysis and disturbances of potassium ion distribution. The handling and administration of plasma is somewhat simpler than that of blood.

3.1642 Grouping of Plasma. It is the contention of some that plasma may be administered without regard to its own blood type or that of the patient. It is doubtless true that the danger of reaction resulting from incompatibility is much less than with blood, for one important source of incompatibility, the cells, is absent. However, until it is absolutely demonstrated that administration of plasma even from individuals having a high titer of agglutinins in their blood is harmless, it would seem safer, as Wiener suggests, to use only plasma of homologous group, or plasma which represents a pool of a number of different groups. Since plasma contains dissolved group substance corresponding to its group (sec. 2.3242, 2.7222) the mixture of plasmas of different groups results in the neutralization of part of the agglutinins of one by the dissolved group substance of the other (23). An even more important factor, probably, is the dilution of the agglutinins which results. Witebsky (44, 45) has proposed the neutralization of the agglutinogens anti-A and anti-B in plasma by the addition of the purified group substances A and B.

* Ravitch, M. M. and A. Blalock, Surg. Gyn. Obst., 1942, **74**: 348.

3.1643 Preparation. The preparation and use of plasma is discussed in quite a number of papers (37, 36, 28, 27). Strumia *et al.* state that plasma taken from blood which has been stored more than 5 days should not be used. Simple directions for the preparation of serum and plasma follow, based partly on the procedure of the Samuel Deutsch Serum Center of the Michael Reese Hospital, Chicago.

3.16431 Serum. Human serum which has been properly prepared does not seem to produce reactions of any serious nature, if its preparation is carefully carried out by trained people.

All material and equipment must be scrupulously clean and sterile. All glassware employed in serum preparation must be washed in chromic acid cleaning solution and thoroughly rinsed in distilled water. All rubber tubing must be properly cleaned by previous boiling in dilute sodium hydroxide and distilled water. After the various parts are assembled they must be wrapped and sterilized in an autoclave. Filters should be cleaned, washed and sterilized promptly after use.

The donors for serum should be healthy, and it is preferable to withdraw the blood from a fasting donor. Allergic individuals should be avoided as blood donors.

Blood aseptically removed from a healthy person is sterile. Proper aseptic technic will maintain the sterility of the blood and resultant serum throughout the various steps of its preparation and will prevent contamination. Adequate sterility tests at various stages of the processing will confirm the absence of contamination.

PROCESSING

Collection of blood.

a. Skin is prepared with iodine and alcohol.

b. Local anesthesia over the vein with 1 drop of 2% novocaine.

c. After venipuncture, blood is withdrawn into the centrifuge bottle.

d. Blood is permitted to clot at room temperature for 20 to 30 minutes and then placed in the refrigerator over night.

Processing of blood.

a. Within 24 hours (not longer), the blood is removed from the refrigerator, and under sterile precautions the clot is separated from the sides of the bottle and a small sample withdrawn for serological test (figure 26).

b. After proper balancing, the bulk blood is centrifuged at 2000 RPM for 20 minutes.

c. The separated serum, plus all loose cells, is withdrawn under vacuum into a sterile centrifuge bottle.

d. This turbid, cell-containing serum is centrifuged at 2000 RPM for 20 minutes to pack the loose cells.

e. The clear supernatant serum is withdrawn into a final sterile bottle and placed in a refrigerator.

Serum processing.

a. The sample of blood withdrawn earlier is tested for syphilis either by a complement fixation or flocculation test, or both.

b. Each specimen of serum prepared is tested for sterility by inoculating 1 cc. of that serum into each of 6 fermentation tubes containing a veal infusion broth (30 to 40 cc.). It is incubated at 37° and observed every two days for one week, for bacterial growth. If there is no bacterial growth, that sample of serum is considered sterile.

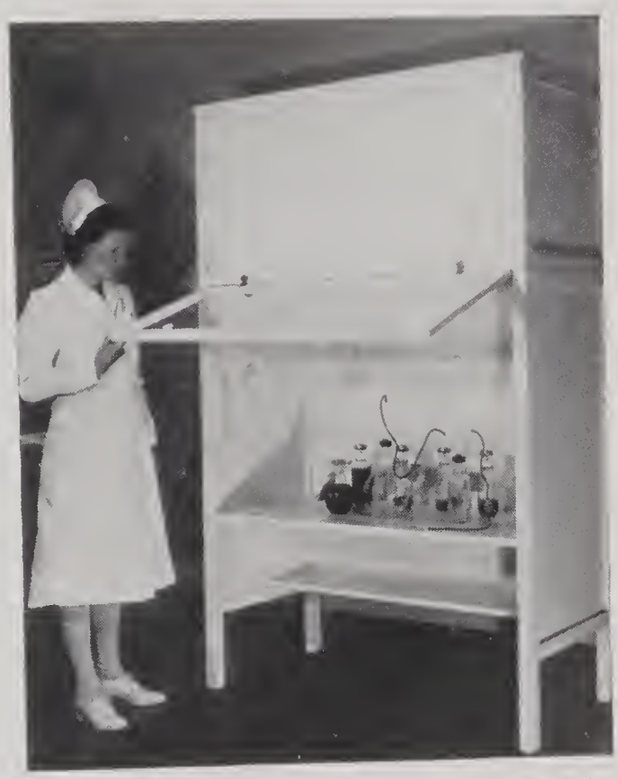


Fig. 26. Plasma transfer cabinet at the Massachusetts Memorial Hospitals (*courtesy of Dr. F. E. Barton, Visiting Surgeon and Director of Transfusion Service*).

c. 30 to 40 specimens of serum, which have been proven serologically negative and sterile, are pooled in a large sterile container.

d. Merthiolate is added to give a final concentration of 1:10,000 and thoroughly mixed.

e. This pooled serum is then filtered through a Berkefeld N filter for clarification.

f. The pooled filtered serum is then transferred to sterile containers in 250 or 500 cc. amounts.

g. During the bottling process, random containers are filled with only 20 cc. of serum, and put aside. 5 cc. of this serum is subsequently withdrawn and inoculated in 6 veal infusion tubes, and the sterility of the pooled serum is checked by incubation of this sample for 7 days. Graded amounts of the serum (5 drops, 10 drops, 1 cc., each for 3 tubes, and the

remainder in the sixth tube) are added to the 6 veal infusion tubes to counteract any bacteriostatic action of the preservative.

3.16432 Plasma. Plasma processing is simpler, especially for any institution employing "preserved blood". As in the preparation of serum, scrupulous cleanliness and sterility of all items of equipment are of paramount importance, and careful checking for sterility is required. Plasma is difficult to filter through a Berkefeld filter because of the presence of fibrinogen, and for that reason cannot be clarified. Furthermore, plasma does not keep as well as serum because fibrin veils and precipitates gradually appear on standing, and since this process of fibrin deposit is a progressive one, clouding of the product cannot be prevented even by filtration. Large particles in serum or plasma render it objectionable for intravenous administration, and if they do appear in plasma which has been preserved for a month or more, the plasma must be filtered to remove the fibrin precipitate and accumulations.

Collection of blood.

a. (As above).

b. (As above).

c. After venipuncture, blood is withdrawn into the container which has in it 50 cc. of 4% sodium citrate solution, until the proper amount (not over 3.5 cc. per pound of body weight (males) or 3.0 cc. per pound (females) is obtained.

d. The blood in the collecting tube is placed in a small pyrex test tube for blood grouping, cross-matching, and serological tests.

e. The citrated blood is placed in a refrigerator and kept at 4°C. until used within 72 to 96 hours.

Processing of plasma.

a. If the blood has not been used within 4 days from the time it is drawn, the supernatant plasma and some free surface cells are withdrawn into a 325 cc. centrifuge bottle.

b. This cell-containing plasma is then centrifuged at 2000 RPM for 20 minutes.

c. The supernatant cell-free plasma is then withdrawn into another sterile bottle. The plasma remaining in the rubber tubing is then seeded into 3 fermentation tubes, and is incubated and observed for sterility.

Pooling of plasma.

a. After a number of specimens of plasma have been proven sterile and serologically negative, they are pooled in a large two-liter bottle.

b. Merthiolate is added so that the final concentration is 1:10,000.

c. The plasma may be stored and used directly from this large container, or it may be used as the filling flask for a number of smaller containers holding from 100 to 500 cc. of plasma in each container. The plasma may

also be withdrawn into containers holding varied amounts of physiological saline, or physiological saline and 5% glucose.

d. Sterility tests, as outlined above, must be made upon the final containers.

3.1644 Dried Plasma. Plasma can be concentrated before use, and methods have been developed for drying plasma, in which state it probably can be preserved indefinitely (28). When properly prepared (37, 10, 13), it is made instantly available by dissolving the dried product in sterile water. By use of less than the original volume of water, concentrated solutions may be obtained. The advantage of having stocks of this valuable material to combat shock in wartime is obvious.

3.165 Purified Plasma Proteins

A purified preparation of any one of the proteins of human plasma could theoretically be used as a substitute for blood in transfusion. In particular, the albumin possesses the advantages of stability and lower molecular weight, which latter point means greater osmotic effect per gram of material. According to Newhouser and Kendrick* "Human albumin, prepared by Cohn† has been given a limited clinical trial and the results thus far are encouraging. Albumin . . . has been administered safely in concentrations up to 30 per cent without untoward reactions and it can be stored in the liquid state without refrigeration. Experimental and clinical trials have proved it to be effective in restoring depleted blood volume resulting from shock and hemorrhage. Since 100 cc. of 30 percent human albumin will provide the same colloidal osmotic pressure, when injected intravenously, as 1,000 cc. of whole blood or 500 cc. of plasma, it is therefore possible to package albumin in a small compact unit ready for immediate use. The fact that albumin can be packaged in small containers is of tremendous importance to our military forces. . . ."

The osmotic function of the plasma proteins is also possessed by other proteins, but intravenous administration of foreign protein is a hazardous proceeding. There might be considerable danger in repeated administration of species-foreign material, because of the possibility of immunizing or sensitizing the patient to the protein, with resulting anaphylactic reaction. The serum globulins seem more dangerous in this respect than serum albumin. If foreign proteins, such as plasma albumin of the cow or horse, could be prepared in pure form and deprived in some way of their antigenicity, such material might be substituted in part for human plasma. It would have the advantage of stability and availability in large quanti-

* *U. S. Naval Med. Bull.* **40:** 1, 1942.

† *Chem. Rev.* **28:** 395, 1941.

ties. Work on this problem is apparently now being undertaken* and may possibly lead to interesting and important results.

3.17 Transport of Blood by Air.†

"THE TIMES lately drew attention to the fleet of aircraft that has been fitted up to carry blood for transfusion to army or civilian hospitals from the depots. A wire crate containing ten bottles of blood is the unit for dispatch. Each bottle of blood has attached to it a tin box containing the sterilized equipment necessary for giving the blood. The insulated boxes for aeroplanes hold two crates, and each box, when loaded, weighs approximately 100 pounds. The box, loaded with two crates is kept in the refrigerator, until just before dispatch. Thereupon an "insert", consisting of a block of ice contained in a tin box, is slipped into the lid of the box. This, combined with the Kapok insulating material with which the walls of the box are lined, ensures that the temperature inside the box will not rise more than 5°C. in 8 hours. Little structural alteration is required in aircraft used for transporting the insulated boxes. The boxes are merely strapped to the fuselage to prevent rolling when in flight. Otherwise, the load is limited only by the carrying power of the aeroplane and the manner in which the load can be distributed. Aircraft of the type used can carry five boxes of blood, that is, 100 pints."

3.18 Serological Investigation of Transfusion Accidents

Transfusion accidents, more or less serious in outcome, continue to occur occasionally. The survey of Levine and Katzin (19) has been referred to above (sec. 3.116).

3.181 General

In cases due to incompatibility, the accompanying circumstances and the clinical symptoms (pain in the lower region during or soon after the transfusion, black coloration of the urine, hemoglobinemia) indicate from the beginning that an unsuitable donor was used. In such cases, a serological examination is made to discover the cause of the incorrect choice of donor. If it was due, for instance, to poor condition or incorrect labeling of the test sera, it is very important to clear up the question quickly, so as to avoid further errors.

Sometimes symptoms are incorrectly ascribed to incompatibility, when the real cause is something else, such as faulty transfusion technic. In such cases proof that the donor was compatible may pave the way for the discovery of the true cause. A special problem is presented by those rare cases where the blood groups are the same but the symptoms nevertheless indicate serological incompatibility. Accidents have occurred with pa-

* *Chem. Rev.* **28**: 395, 1941.

† From *The Lancet*, Dec. 30, 1939, p. 1378.

tients who have previously received numerous transfusions, often from the same donor, and tolerated them well. In such cases the behavior of the bloods on cross-matching is particularly important. The strength of any reaction observed, and whether it takes place at 37°, must be noticed. The possibility that the later reactions were due to anti-Rh antibodies should be kept in mind (42, 22, 41), for it now seems not unlikely that a considerable portion of these reactions are due to the transfusion of Rh+ blood into Rh− patients.

Investigation of transfusion accidents may also be important if there is any question of liability involved, and it should be kept in mind that the data may later be required in court.

Usually the serological investigation of a transfusion accident yields an unequivocal result: either donor and patient were serologically compatible or they were not compatible. The last result does not in itself prove that the accident was caused by the serological discrepancy, for we know that serologically "incompatible" blood can at times be transfused, even in fairly large amounts, without producing any untoward symptoms. It must be shown in addition that the actual symptoms were those typical of transfusion accidents due to incompatibility.

In order to be able to undertake the necessary investigations should they be required, it is the rule in our laboratories to keep all blood samples sent in for group determinations in the ice box for a week before discarding them. For it must be kept in mind that the patient's blood may be temporarily altered by the transfusion (e.g., the agglutinins might be absorbed); also, new samples cannot always be obtained. While checking the possibility that samples were interchanged, it is desirable to test at the same time fresh samples, if they are obtainable. The blood group of a dead person can still be determined at autopsy, or even later from anatomical or pathological material preserved in formalin, as will be discussed below (sec. 3.421). Moureau was able in a case of death following transfusion to determine the blood group of the patient a year later by investigation of his relatives, and thus make clear the circumstances of the accident.

3.182 Procedure

a. Check the identity of the blood samples. Take fresh samples of definitely known identity, if possible.

b. Prepare or test new test reagents (including immune sera, especially for the test for A), of known strength and specificity.

c. Test the samples from donor and recipient. Redetermine the blood groups, checking both cell and serum characteristics (sec. 3.117).

d. Repeat cross-matching (sec. 3.1172). Make tests at 37°, 20°, and 0°.

e. Include in the tests controls: *sera* and *cells* of individuals of all groups,

including several samples of group O. Test patient's and donor's *cells* with several samples of serum of their own group and with AB(o) serum.

f. Test patient and donor for the Rh factor (sec. 2.62), and if the patient is Rh−, test his serum for anti-Rh agglutinins (sec. 2.62).

g. If no demonstrable hemolysis occurred in the patient's circulation, determine the M, N types of donor and patient. If these are different, it may be tested if the donor's cells have persisted in the circulation after the transfusion, as they should have (sec. 3.2). If none of the donor's cells are left, incompatibility in regard to some factor, probably Rh, was present. This incompatibility will not always be followed by the production of demonstrable anti-Rh antibodies in the patient's serum (41).

According to Wiener (41), if the patient's serum is tested too soon, or too late, after the transfusion, or if the bloods are sent by mail, anti-Rh antibodies may be missed.

3.19 Regulations Governing Blood Donors and Blood Banks in New York City*

Section 108 of the Sanitary code states: "*Blood donors regulated; blood donors, professional and voluntary blood donors defined; registration of professional blood donors; blood banks regulated.* No person shall act as and no physician, hospital or institution shall use, a blood donor, either professional or voluntary, in the City of New York otherwise than in accordance with the regulations of the Board of Health, and no person shall act as a professional blood donor in said city without a certificate of registration issued by the Department of Health.

"No blood bank shall be maintained or operated in the City of New York other than in hospitals in which there is a clinical laboratory under permit of the Board of Health for bacteriology, blood typing and serology, or otherwise than in accordance with the regulations of the Board of Health.

"'*Blood donor' defined.* The term 'blood donor' as used herein shall be taken to mean and include any person who holds himself out as willing to dispose of his blood, or who offers his blood, or whose blood is used for transfusion purposes either by direct introduction or by storage and subsequent introduction into the blood-vascular system or any other person.

"'*Professional blood donor' defined.* The term 'professional blood donor' as used herein shall be taken to mean and include a blood donor who offers or gives his blood for a fee.

"'*Voluntary blood donor' defined.* The term 'voluntary blood donor' as used herein shall be taken to mean and include a blood donor who offers or gives his blood gratuitously or without a fee.

"'*Blood banks' defined.* The term 'blood bank' as used herein shall be

* Reproduced by permission of the Department of Health, New York City.

taken to mean and include any system of storage of human blood for subsequent use for transfusion purposes.

"This section as amended shall take effect immediately, and any person heretofore registered as a blood donor and until the expiration of his certificate of registration shall be deemed a professional blood donor. (Adopted November 21, 1930 and amended March 14, 1939).

"Regulation 1. *Professional blood donor's application and certificate of registration.*

"(a) Application for a certificate of registration as a professional blood donor shall be made upon official forms furnished for such purpose by the Department of Health. At the time of registration the applicant shall submit to a careful physical examination of the following organs: skin, mouth, pharynx, heart, lungs, abdomen (particularly the liver and spleen), rectum, genitalia and lymphatic glands, and submit a certificate on the form provided by the Department of Health, from a duly licensed physician of the State of New York showing that such an examination was had by him within seven days of the date of his application for registration.

"(b) No person shall be accepted for registration unless he shall approximate an average weight for height, according to the standard tables of normal average of blood and health; his systolic blood pressure shall be at least 110 mm of mercury.

"(c) No person shall be accepted for registration unless a specimen of his blood, taken at the time of his application and tested by the Department of Health with the Wassermann or other approved serological test for syphilis shall react negatively to such test.

"(d) No person shall be accepted for registration who exhibits suspicious scars or symptoms of syphilis, gonorrhea or other venereal disease, who gives a history of syphilis or malarial infection, or who presents evidence of heart disease, hyperthyroidism, asthma, tuberculosis or any other communicable disease, evidence of drug addiction or who has any obvious infection of the teeth or gums with suppurative lesions.

"(e) No person shall be accepted for registration until a determination of his blood grouping has been made in accordance with Regulation 2 herein, showing in which group he is classified.

"(f) In addition to the aforementioned requirements, the Commissioner of Health shall have the right to deny registration to anyone applying for registration as a professional blood donor, when in his opinion the condition or history of the said applicant is such that transfusion of blood from said applicant might be detrimental to the life or health of a recipient.

"(g) The certificate of registration as a professional blood donor shall expire one year from the date of issuance.

"Regulation 2. *Grouping of professional blood donors.* The blood group

of each professional blood donor shall be established by suitable tests performed at laboratories under permit of the Board or Department of Health by testing his blood with known group specific sera and also by testing the professional blood donor's serum against known Group A and Group B corpuscles (international classification).

"Regulation 3. *Professional blood donor's identification and personal record book.* Every professional blood donor shall have and keep on his person an identification and personal record book issued by the Department of Health in which shall appear the seal of the Health Department of the City of New York together with the date of registration. Such personal record book shall contain a personal description of the professional blood donor, his photograph and signature, and shall contain sufficient space for the entries therein of the serological test for syphilis by the Department of Health, for the medical report of his physical examination, and for the blood grouping of his blood, together with the date of such grouping and the name and address of the director of the laboratory wherein the said grouping was performed. The personal record book of every professional blood donor shall also contain a record of each transfusion in which he has participated and the following facts for each transfusion shall be entered therein by the physician making the transfusion or his representative; the date of the transfusion, the place where the transfusion occurred, the quantity of blood given and the name of the physician who made the transfusion. There shall also be entered the result of the physical examination of the professional donor, the hemoglobin percent of his blood, the result of the serological test for syphilis and the cross-agglutination test, as required in Regulation 5.

"Regulation 4. *Suspension or revocation of certificate of registration.* In event that any registered professional blood donor shall be shown to have a positive reaction to any approved serological test for syphilis, or where the examination of his sputum indicates the presence of tubercle bacilli, or where he is shown to have a history of malarial infection, or evidence of heart disease or typhoid fever, or any communicable disease, or any of the conditions mentioned in Regulation 1 herein, or where his personal record indicates that he has already given an excessive quantity of blood, the physician making such examination is hereby authorized to collect and forward immediately the professional blood donor's registration book to the Department of Health. In such case the professional blood donor shall surrender his book to the physician. In the event that the physician does not collect and forward the blood donor's book to the Health Department, the physician shall notify immediately said Department of the circumstances. The Commissioner of Health may revoke such certificate of registration for any of the above mentioned conditions or for other cause.

"Regulation 5. *Physical and serological examination of all professional and voluntary blood donors required immediately prior to transfusion.* Immediately prior to each transfusion, or collecting of blood subsequently to be used for transfusion purposes, every blood donor, whether professional or voluntary, shall submit to a physical examination by a physician on whom has been conferred the degree of doctor of medicine. Such an examination shall have special reference to the organs, tissues and conditions mentioned in Regulation 1 hereof. No blood donor shall be used for transfusion purposes who exhibits suspicious scars or symptoms of syphilis, gonorrhea or other venereal disease, or who gives a history of syphilis or malarial infection, or who presents evidence of heart disease, hyperthyroidism, asthma, tuberculosis, venereal disease or any other communicable disease, evidence of drug addiction, or who has any obvious infection of the teeth or gums with suppurative lesions. Such an examination shall also include a determination of the hemoglobin content of the donor's blood by means of a standardized or generally accepted hemoglobinometer, and no blood donor shall be accepted whose hemoglobin is found to be less than 85 percent of the normal for the method employed. Such examination shall further include a serological test for syphilis approved by the Department of Health, and cross matching of the donor's with the recipient's blood. No blood donor shall be used for transfusion purposes whose blood reacts positively to such a test for syphilis, or where there is demonstrated incompatibility between the blood of the donor and that of the recipient. The pulse and temperature of the blood donor shall also be taken and any indication that the donor's condition is not normal shall debar such donor from service at that time.

"Regulation 6. *Hospitals, institutions and physicians to keep record of transfusion.* The superintendent or person in charge of every hospital or institution shall keep a record of every blood transfusion performed in such hospital or institution, and the said record shall show:

(a) The name of the physician or surgeon making the transfusion.
(b) The name of the donor, and if a professional donor, the registration number.
(c) The blood grouping classification.
(d) The hemoglobin percent.
(e) The results of the physical examination, of the serological test for syphilis of the donor, and of the cross matching test prior to transfusion.
(f) The quantity of blood given.
(g) The name of the patient.
(h) The date of the transfusion and any untoward reaction which may have occurred.

"A similar record shall be kept by a physician or surgeon of every transfusion performed by him outside of a hospital or institution. Such records shall be open to inspection by an inspector or other duly authorized representative of the Department of Health.

"Regulation 7. *Untoward reactions to be reported.* It shall be the duty of the superintendent or person in charge of every hospital or institution, as well as of every private physician, to report promptly to the Department of Health all instances in which a transfusion has been accompanied or followed by any blood stream infection or evidence of hemolysis such as hemoglobinuria, jaundice or death, associated with the transfusion. Such report shall give the name of the patient, date of transfusion, name of the donor, name of the physician in charge of the transfusion and any other pertinent facts.

"Regulation 8. *Transfusions in dire emergencies.* No transfusion shall be made otherwise than in accordance with these regulations, except in dire emergency. Such dire emergency shall be deemed to exist only when it can be presumed that the death of the patient would occur while awaiting the report of the cross-agglutination test or serological test for syphilis. No transfusion, however, shall take place in a case of dire emergency until a physical examination of the donor has been made and specimens of blood taken for a serological test for syphilis and for cross matching of the donor's and recipient's blood, which specimens shall be examined as soon as possible after the transfusion.

"Regulation 9. *Blood banks for transfusion purposes regulated; records to be kept*:

"(a) Blood collected and stored for subsequent use for transfusion purposes shall comply with the following conditions:

(1) The blood shall be collected by a physician upon whom has been conferred the degree of doctor of medicine, or under his direction.

(2) The blood shall be collected from a donor who has met all the requirements governing blood donors contained in these regulations.

(3) The blood shall be collected aseptically into a sterilized container and treated with a proper anti-coagulant.

(4) Additional blood specimens shall be collected from the donor and placed in separate tubes for the typing and serological tests and for subsequent cross matching tests with the recipient's blood. Such tubes shall be immediately and properly labeled for identification purposes, and the tube or tubes for subsequent cross matching tests shall be securely fastened to the container of the blood.

(5) The blood shall have a hemoglobin content of not less than eighty-five per cent (85%) of the normal for the method employed.

(6) The blood shall have a white blood cell count not in excess of 10,000 white blood cells per cubic millimeter.

(7) The blood shall react negatively to an approved serological test for syphilis.

(8) The container of blood shall be securely sealed and remain sealed until used for transfusion or destroyed or disposed of for a purpose other than transfusion. It shall be legibly labeled immediately after collection of the blood with the date of collection, the donor's name, address, age, sex and color, the signature of the physician collecting the blood or under whose direction the blood was collected, and with the results of the blood grouping test and serological test for syphilis, as soon as the results of such tests have been reported.

(9) The blood shall be stored aseptically and refrigerated at not more than five (5) degrees centigrade until immediately before use.

(10) The blood shall not be used if it shows, at any time, evidence of hemolysis.

(11) The blood shall not be used later than eighteen (18) days after collection.

(12) Where blood has been placed in the refrigerator pending the tests required herein, and the result of any such test indicates that the blood does not conform with any of the requirements of this regulation, the same shall be immediately removed from the refrigerator and destroyed or disposed of for a purpose other than transfusion.

(13) Blood which shows evidence of hemolysis or is over-aged, as stated in paragraph 11 herein, shall be immediately removed from the refrigerator and destroyed or disposed of for a purpose other than transfusion.

"(b) Adequate and complete records of all specimens of blood stored in a blood bank shall be kept and shall be available at all times for inspection by an authorized representative of the Department of Health. These records shall include the following:

(1) The date of collection, the amount collected, and the name of the physician collecting the blood or under whose direction the blood was collected.

(2) The donor's name, address, age, sex, color, registration number if a professional blood donor, and the results of the physical examination.

(3) The results of the blood grouping tests, serological tests for syphilis, the hemoglobin determination, and the white blood cell count.

(4) An entry showing the disposition of such blood and if used for a transfusion the quantity of blood given, the name of the patient, the date of the transfusion, the result of the cross matching test, and any untoward reaction which may have occurred."

(Adopted November 21, 1930 and amended March 14, 1939).

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3.2 Serological Determination of Survival of Transfused Blood

3.21 Principle

Under favorable conditions, the blood corpuscles of the donor can be observed in the blood of the patient after transfusion. Use has actually been made of a constitutional difference in the shapes of the erythrocytes for this purpose, but more commonly the end is attained by serological methods. If there is a serological difference between the cells of the donor and those of the recipient, they may be differentiated by use of a serum which agglutinates only one of them. For example, if the patient belongs to group A and the donor to group O, an anti-A serum would be used. Sufficiently strong serum (one which leaves no A cells unagglutinated) must be available. When blood is taken from the recipient after the transfusion, and mixed with such a serum, if donor's blood is still present there will be seen, in addition to the agglutinated blood of the patient, some unagglutinated cells, which are those of the donor.

This principle was first applied by Todd and White (5) to study the survival of transfused erythrocytes in cattle. The method was applied to the study of transfusions in man by Ashby (1). Ashby made use of patients of groups A, B, and AB who had received blood from so-called universal donors (group O). She counted the numbers of agglutinated cells in mixtures of the recipient's blood and homologous strong agglutinin, and by following the gradual disappearance of the donor's cells, was able to determine that the introduced cells survived for periods up to 80, to 120 days. Similar results were found by Wearn *et al.* (6).

If we consider only the four blood groups, the length of survival of transfused cells can be tested only when the donor is of a different group from the recipient. Since we employ today almost exclusively donors of the

same group, we have to use serological methods which are independent of the four blood groups to distinguish the cells of two persons. This is possible in many cases by using the M and N factors. Employing immune agglutinating sera for M and N, Landsteiner, Levine and Janes (3), Wiener (7), and Dekkers (2), confirmed the previous estimates for the survival of transfused cells. Wiener and Schaefer (9) applied this technic to a study of the length of life of transfused cells from stored blood, and were able to show that cells stored more than a few days disappeared from the patient's circulation sooner than fresh blood, and blood stored for as long as 21 days survived only about 24 hours.

Transfusions to persons of groups A and AB might perhaps be followed serologically by using blood of different subgroups.

To summarize, the following combinations of donor and recipient could be used to detect the survival of transfused cells:

3.211 The Four Blood Groups

a. The patient belongs to one of the 3 groups, A, B, or AB, and the donor to group O.

b. The patient belongs to group AB, and the donor to one of the other 3 groups.

Since the use of universal donors and universal recipients as such has now been practically abandoned, only rarely is there occasion to make use of one of these combinations.

3.212 Subgroups of A and AB

a. Patient belongs to subgroup A_1 or A_1B, donor to A_2 or A_2B, respectively.

b. The reverse of a.

Wiener (8) has made use of the subgroups in this way, detecting transfused A_1 cells with B serum. He states that only certain absorbed B sera are suitable, as some give partial agglutination with other bloods.

The slightly greater difficulty in preparing the sera is perhaps a disadvantage in using these combinations.

3.213 The M and N Factors

The following combinations can be used, irrespective of which is the donor and which the recipient:

Combination	First person	Second person
a	M	N
b	M	MN
c	N	MN

These bloods could be differentiated in the following way:

Combination	Persons	Cells react with serum anti-M	anti-N
a	M	+	−
	N	−	+
b	M	(+)	−
	MN	(+)	+
c	N	−	(+)
	MN	+	(+)

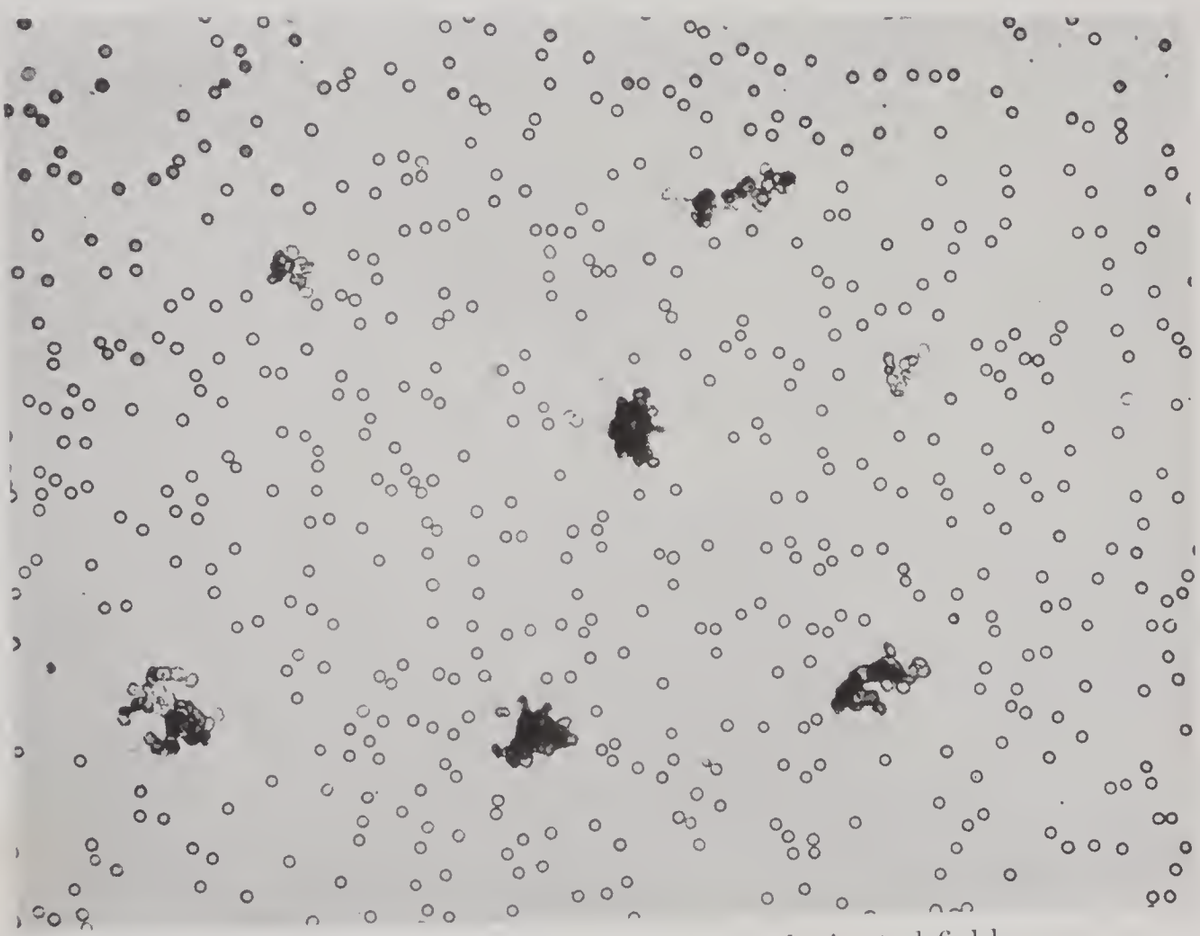

Fig. 27. Agglutinated cells in unagglutinated field

According to the blood composition of the donor, his blood will easily be recognized either by agglutination or failure to agglutinate. In combination a, where one person is M and the other N, the cells of either donor or recipient can be recognized by a positive reaction.

3.22 Procedure

3.221 Qualitative

A drop of the blood suspension is mixed with a drop of the agglutinating serum, mixed well, and covered with a cover slip (figure 27). The illustration shows a successful differentiation of the blood of a donor of type M in the blood of a patient of type N. The tests were carried out with a

strong absorbed anti-M serum. A control test with the donor's cells alone showed complete agglutination.

A number of workers, including Wiener and Schaefer (9) and Levine (4) have used the M, N types in this way.

These procedures are purely qualitative, and are adequate for most practical purposes, where it is sufficient simply to determine whether any foreign cells remain. By testing at the same time artificial mixtures of the two kinds of blood in different proportions, it is possible to estimate the approximate per cent of foreign cells present. Since the agglutinated corpuscles settle more quickly, it is possible by this means to separate the two kinds of cells in the test tube.

3.222 Quantitative

The quantitative technic enables a more precise measure of the degree of survival to be made. Wiener and Schaefer (9) have described their procedure as follows: Following a transfusion of type N blood to a type M or MN patient, for example, a sample of blood is taken from the finger into a red cell pipette, using anti-M testing fluid as the diluting fluid. The mixture is allowed to stand for one or two hours until agglutination is complete, and then gently shaken. A drop of fluid is transferred to a counting chamber, avoiding clumps as much as possible. The unagglutinated cells, which are the donor's cells, can now be counted in the usual manner.

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3.3 Problems of Relationship, Including Disputed Paternity

3.31 Principle

Since the laws of heredity governing the A, B blood groups and the M, N types are accurately known, from a knowledge of the blood groups of parents it is possible to state what blood group or groups their children must belong to. Clearly this means that blood group determinations will sometimes provide us with information which will bear on problems of relationship. The most frequently occurring of these problems is, of

course, the dispute which often arises regarding the paternity of an illegitimate child.

Figure 28 shows schematically how men of certain blood groups can be ruled out as possible fathers, in one of the possible cases. Their exclusion is based on the principle that the blood group substance B (or A) cannot arise spontaneously in an individual, but must come from one or both of his parents. For a presentation of this subject from the legal point of view, see Wigmore (29). It is of interest to note that this authority on evidence is of the opinion that the court has ample right to order an individual to submit to these tests, stating (vol. VIII, sec. 2220) ". . . the use of the *blood-group test* for *paternity* may require the denial of any privilege."*

In order that the bearing of blood groups on such problems may be appreciated even by those who are not physicians, serologists, or geneticists, it will be necessary to explain here the mode of inheritance of the blood groups and types, starting with considerations which may seem elementary to the initiated.

Although it is hoped that the principle of these procedures can be understood by all, the actual tests, naturally, should be entrusted only to experts.

3.32 Blood Grouping Expert Defined

Certain facts suggest that the proper definition of the term "blood grouping expert" has not always been understood. The present authors, being not unfamiliar with the subject, may perhaps be allowed to suggest a tentative definition here.

A blood grouping expert is a person with adequate scientific training, such as is symbolized by the degrees of PhD., Sc.D., M.D., or equivalents, who for a number of years has been carrying out blood grouping tests, and conducting research in serology as applied to blood groups.

It should be emphasized that the essential thing is familiarity with the subject, and not possession of academic degrees, or occupation of an important academic or administrative post. A man may be a professor of immunology, hematology, or other related subjects, and may have a life-time of research in such subjects behind him, without being a blood grouping expert. In particular it should be emphasized that there is nothing specifically medical about the subject, and the average physician is decidedly not qualified to carry out forensic blood grouping tests, and a good many non-medical scientists are, since research in blood grouping

* From *A Treatise on Evidence*, by J. H. Wigmore, by permission of the author and Little, Brown and Co., publishers. See also *Beach v. Beach* in Federal Reporter, Oct. 28, 1940, p. 479.

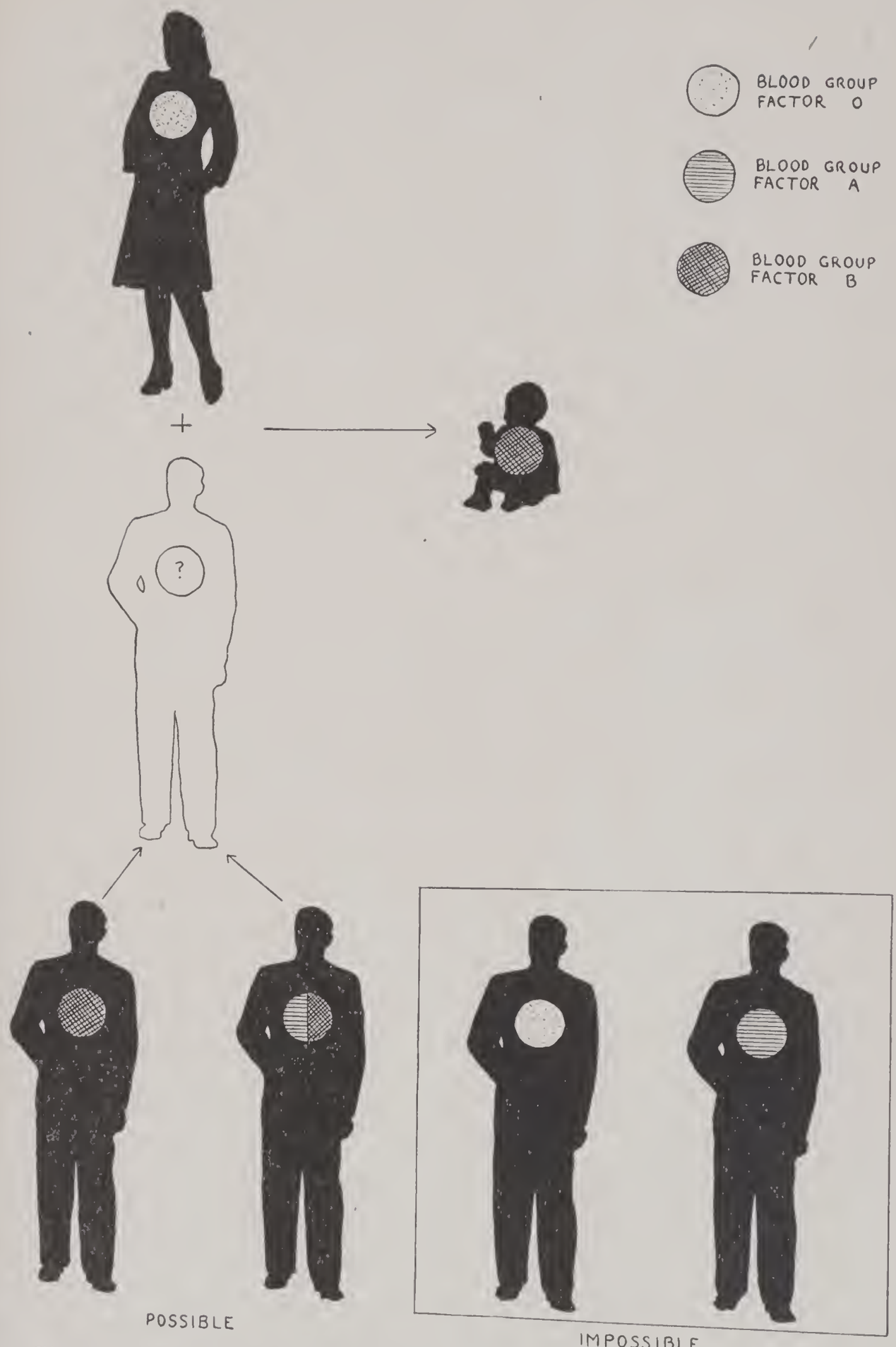

Fig. 28. Example of the application of blood tests to problems of paternity

may be approached from many angles, genetical, biochemical, and others. This point is often misunderstood by members of the legal fraternity.

The most important proof of mastery of a subject is the publication of original research dealing with it, in reputable scientific journals. So an expert should preferably be able to point to three or more original investigations regarding blood grouping, which have been published in the usual journals. This point also is not always understood by those dealing with legal matters, and there is sometimes a tendency to regard the individual as no expert unless he is the director of a large laboratory, with so many helpers working under him that he never carries out the tests himself.

Finally, although this is not always an easy point to ascertain, the opinion of the generally recognized authorities in the field, regarding the particular individual in question, is of value in establishing his competence. Is his work mentioned in any of their scientific publications, articles in scientific journals, or books? Such recognition is of course not essential to the establishment of the competence of an expert, but will be of value in case of dispute.*

3.33 Genetic Principles

An elementary knowledge of the laws of genetics will make it easier to understand the following exposition, but it will by no means be found essential. It should be clearly understood that the mode of inheritance of the blood groups is a *fact*, established by tens of thousands of recorded observations. The purpose of introducing genetic theory in connection with this exposition is only to make the laws clearer and to fix them in mind (12).

Geneticists have shown that the hereditary characters of animals and plants are determined by units localized in submicroscopic structures called "genes". These in turn are supposed to occur in pairs, and to be localized in small but microscopically visible rod-like bodies that occur in the nuclei of the cells of which plant and animal bodies are built up, and by which they are propogated. These rods are called "chromosomes" and are observed to occur in pairs, in conformity with theory. A chromosome may carry a number of genes. One member of a pair of chromosomes caries at each point of its structure a gene corresponding to the one at that same point in the other member of the pair.

During the formation of the cells concerned in reproduction (sperm and ova), these pairs of chromosomes separate, and each sperm or ovum contains only one of each kind of chromosome, and forms an exception

* Since the above was written, Wiener (27) has proposed a very similar definition. He further suggests that anyone seeking qualification as an expert pass a practical examination by grouping several known bloods correctly, and that only workers who prepare their own sera be considered.

to the general rule that each cell contains a pair of each kind. Figure 29 may assist in making this clear.

In very numerous experiments on many varieties of living things propagated by sexual reproduction, it has been observed that hereditary characters occur in pairs, whose relationship is shown by breeding experiments just as this chromosomal theory would demand. The human blood groups are inherited by such a mechanism. They are predetermined by the presence or absence in the chromosomes of two factors (or genes) called "A" and "B". Absence of A and B may be indicated by "O". Since each body cell has a pair of chromosomes, each of which carries or fails to carry one of these factors, an individual's genetic constitution may be represented by AB, AA, AO, BB, BO, or OO, where O represents the absence in the chromosome of either the A or B factor at that point.

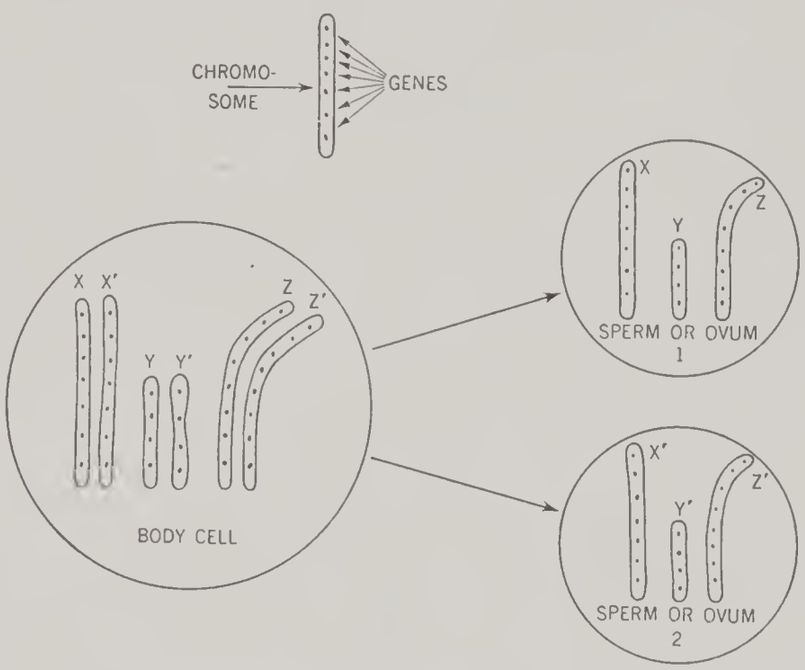

Fig. 29. Separation of chromosome pairs in formation of gametes

From this it is easy to see that the individual whose genetic formula (genotype) is AB will exhibit both of these factors in his blood (and other) cells, while the AA individual will have only the A characteristic, and the OO will have neither A nor B.

The thing that is tested for in the blood cells is not the gene itself, but the substances A and B produced under the specific influence of the inherited genes. The fact that the AO individual has only one dose, so to speak, of the A factor makes no practical difference, and his blood is routinely indistinguishable, by direct test, from that of the AA individual. (Compare, however, sec. 2.511). This means that we have but four types of blood (phenotypes), O, A, B, and AB, distinguishable from each other by ordinary laboratory tests. These are shown in table 24, together with the corresponding designations in the two other (obsolescent) systems of

nomenclature. These earlier systems of naming are still unfortunately used to some extent in certain hospitals.

Knowing that mature reproductive cells (gametes) of each sex emerge with only one chromosome of a pair, and consequently only one gene of a pair, we can mathematically predict the way in which the blood groups will be inherited. When sperm and ovum merge it is readily understood that the resulting cell contains its set of pairs of chromosomes; each parent has contributed one member of each pair.

A man of group AB produces two types of sperm, one containing the factor A and one containing the factor B. These are produced on the average in equal numbers. A woman of group AB will produce two types of ovum, one containing A and one containing B. If such a man and woman mate, it will be a matter of even chances whether an A sperm fertilizes a B or an A ovum, and whether a B sperm fertilizes a B or an A ovum, so that three types of offspring could be produced, AB, AA, BB, BA.

TABLE 24

DESIGNATION OF BLOOD GROUPS AND THEIR GENETIC FORMULAS

International name (Phenotype)	Jansky name	Moss name	Genetic constitution (genotype)
O	I	IV	OO
A	II	II	AA or AO
B	III	III	BB or BO
AB	IV	I	AB

(The AB and BA offspring will be just alike, since each will have both substances in his blood.) The percentage of offspring, if we observe a statistically large enough number of such matings (or of children of one such mating), will be 25 per cent A, 25 per cent B, and 50 per cent AB. It is conventional in genetic literature to illustrate such a process of reasoning about the results of a mating by the checkerboard diagrams shown in table 25. (Either factor shown on the left may combine genetically with either of the two at the top of each diagram).

When we consider a mating involving an individual of group A or B (phenotype), we must remember that there are really two genotypes in each group; e.g. in A, one whose genetic constitution is AA (homozygous), and another whose constitution is AO (heterozygous). The first will produce only one kind of gamete (or reproductive cell), all containing A, while the other will produce two types of gamete, 50 per cent containing A and 50 per cent not containing A.

Only four of the 21 possible kinds of mating are shown in table 25. Column III shows how a child's group may differ from that of either

parent; column IV shows why an O parent can not have an AB child, or, that an O child can not descend from an AB parent, and vice versa. The reader will easily be able to work out the other 17 possible types of mating for himself, and thus verify table 26. (They are fully worked out for each of the 21 combinations in (29).)

Thousands of families have been tested to check the heredity of the blood groups. In this connection a point arises which is sometimes presented incorrectly in the courts: will we expect to find every family obeys these rules exactly, and that there are *no* exceptions? The answer is, yes and no. The reply cannot be an unqualified yes for two reasons. (1) Not

TABLE 25

DIAGRAMMATIC EXAMPLES OF THE HEREDITARY TRANSMISSION OF THE FACTORS THAT DETERMINE CERTAIN BLOOD GROUPS

I Mating AA x AA	II AA x AO	III AO x BO	IV OO x AB
A | A A A | AA AA A | AA AA	A | A O A | AA AO A | AA AO	B | O A | AB AO O | BO OO	A | B O | AO BO O | AO BO
Progeny: 100% group A	100% A	25% O 25% A 25% B 25% AB	50% A 50% B

On the top of each diagram are designated the factors possessed (and transmissible) by one parent; on the left, those of the other; which parent does not matter, because this transmissibility has been shown to be independent of sex. The letters within the horizontal lines show the possible progeny resulting.

The fundamental law is that a factor can not be present in the blood of a child unless it was present in the blood of at least one of its parents (9).

Diagrams of all the 21 possible types of mating are tabulated by (29).

every worker who has reported on blood group tests has used an irreproachable technic; indeed it is clear that some have used a deplorable technic. Also, before the laws governing heredity of the blood groups, as outlined above, were understood, certain errors were not known to constitute exceptions to the rules, and the people were not retested. (2) In any large group of children taken at random, a number will be illegitimate, and consequently may not conform to the blood group of their supposed fathers.

3.331 The Two Laws of Blood Group Inheritance

Our present knowledge of the inheritance of the A, B blood groups was discovered in two steps: von Dungern and Hirszfeld (9) in 1910

demonstrated that the agglutinogens A and B are inherited as simple Mendelian dominants, a fact which we have just paraphrased by stating that O represents the absence of A or B. In 1924, Bernstein (2, 3) showed that the three genes, O, A, and B formed a series of triple allelomorphs, and proposed the mechanism of inheritance we have just outlined, and which is now known to be correct.

TABLE 26

BLOOD GROUPS OF OFFSPRING POSSIBLE OR IMPOSSIBLE FROM ANY MATING COMBINATION

Mating combination	Alleged father	Known mother	Children possible from their mating	Children not possible from their mating. Decisive for non-paterntity	Impossible from this mother in any mating
1	O	O	O	A, B, (AB)	AB
2	O	A	O, A	B, AB	
3	O	B	O, B	A, AB	
4	O	AB	A, B	AB (O)	O
5	A	O	O, A	B, (AB)	AB
6	A	A	O, A	B, AB	
7	A	B	O, A, B, AB		
8	A	AB	A, B, AB	(O)	O
9	B	O	O, B	A, (AB)	AB
10	B	A	O, A, B, AB		
11	B	B	O, B	A, AB	
12	B	AB	B, A, AB	(O)	O
13	AB	O	A, B	O, (AB)	AB
14	AB	A	A, B, AB	O	
15	AB	B	A, B, AB	O	
16	AB	AB	A, B, AB	(O)	O

The letters designate the blood groups of the respective individuals (see table 24). Those in parentheses (column 5) could not be children of the corresponding mothers (column 3) in any mating. Since no such child could exist to raise a problem of proof, these instances are omitted from table 29 which summarizes the net indications of non-paternity deducible from these blood groupings.

3.34 Results of Studies of Families

If we now examine the results of studies on the inheritance of blood groups in families, we find the sequence of discovery we have just referred to reflected in the data. Between 1910 and 1924, a number of exceptions to the Bernstein rules were reported, but since these rules had not yet been published, the various authors probably did not reexamine the bloods of these "exceptions" with the care which was used after 1924. We find

that after 1924 the number of such exceptions dropped markedly. For this reason Wiener, in presenting studies on the heredity of the A, B groups (25), presents only data published after 1924, and includes only studies in which 250 or more children were tested. It has seemed advisable to us to follow this example; consequently, our table 27 contains the data summarized by Wiener, plus the data of Dahr (6), Dahr and Buszmann (7), Dahr, Offe and Weber (8), Matta (16), and Moureau (17). It summarizes the results of experimental tests of the laws of heredity as they apply to blood groups.

3.341 "Exceptions" to the First Law. The numbers in parentheses in table 27 represent apparent exceptions to the rules of inheritance as

TABLE 27

SUMMARY OF STUDIES ON HEREDITY OF THE BLOOD GROUPS

(Conducted by workers aware of the Bernstein hypothesis, and involving the study of 250 or more children)

Groups of parents	Number of families	Number of children in group				
		O	A	B	AB	Total
O x O	1405	3355	(14)	(9)	0	3378
O x A	2647	2486	3389	(10)	(2)	5887
O x B	1365	1315	(7)	1690	(1)	3013
O x AB	504	(6)	607	612	(3)	1228
A x A	1270	516	2354	0	(1)	2871
A x B	1299	561	908	734	782	2985
A x AB	419	0	478	238	279	995
B x B	536	188	(1)	975	0	1164
B x AB	304	(2)	171	383	225	781
AB x AB	57	0	26	36	63	125
Totals.	9806	8429	7955	4687	1356	22,427

The numbers in parentheses represent "exceptions" (see text) to the laws of inheritance of von Dungern and Hirszfeld and of Bernstein.

discovered by von Dungern and Hirszfeld and by Bernstein. There are only 45 exceptions to the rules of the former, out of the total of 22,427 children, and in not one of these cases could the possibility of illegitimacy be excluded. *There is not a single completely established exception to the law that A and B do not appear in the blood of a child unless present in the blood of at least one of the parents.*

If in addition to illegitimacy as a cause of the "exceptions" observed, faulty technic were also a cause, we should expect a marked decrease in exceptions to the now accepted Bernstein laws, after 1925. That this is indeed the case is shown in table 28. It is readily understandable that

mistaking a group A_2B (or A_3B) parent for group B, for instance, might lead to apparent exceptions if the family contained children of group A.

It seems clear that in future investigations, discrepancies will continue to be fewer than in the early years of this work. It would seem, however, that some exceptions will continue to be found. We may confidently ascribe practically all of these to illegitimacy. Since blood group tests can detect on the average about one sixth of all illegitimacies, this implies about 12 illegitimate children per 1000, a credible figure (20, 11).

3.342 "Exceptions" to the Second Law

Bernstein's theory that the genes O, A, and B form a series of triple allelomorphs has as a consequence the rule that a child of group O cannot come from a father or mother of group AB, and a child of group AB can not come from a father or mother of group O (see table 25, above). Data are available to test this statement (table 27). Before we proceed to do so

TABLE 28

RELATIVE NUMBERS OF EXCEPTIONS TO THE BERNSTEIN THEORY OBSERVED IN DIFFERENT PERIODS (MODIFIED AFTER LATTES)

Data for period	Number of families	Number of children	Exceptions per 1000 families	Exceptions per 1000 children
1910–1925	973	2270	19.5	13.2
1926	928	2213	1.1	0.47
1927–1930	5018	11,141	4	2
1935–1937	541	1473	3.7	2.0

let us consider that (1) children of group O with AB father, or vice versa, will not be conclusive evidence of an exception, because of the possibility of illegitimacy; (2) the existence of the weaker subgroups of A (A_2, A_3, etc.), and the fact that their reactivity may be particularly weak in very young children, has probably led to a number of errors in grouping.

Worsaae (30) reported an apparent exception to the second (Bernstein) law, where a mother belonged to group AB (subgroup A_2B) and had a child apparently of group O. A few months after birth, however, the child's group could be demonstrated to be really A_2. Haselhorst and Lauer (10) observed a similar case in which the infant remained a group O, and this seems to constitute a real exception. (Other apparent exceptions, such as that of Kossovitch (14) have not been rechecked sufficiently, and are not considered, even by their discoverers, to constitute real exceptions).

It is probably worth mentioning that this child (born June 16, 1928) is a deaf-mute, nearly completely blind, and has the left side of the face deformed (17, 10). These pathological symptoms have led a number of

authors to question whether we can consider this a valid exception to the rules of heredity in *normal* individuals, rather than some abnormal development due to some chromosomal change during the development of the fertilized ovum (23, 10).

Such exceptions might perhaps result from mutations (25) or from non-disjunction (Levine).

Even should there routinely occur one exception in every thousand or several thousand cases, blood grouping evidence would still be far more reliable than, for instance, eye-witness testimony, which is routinely admitted as evidence in court cases. We may recall as one instance of many the case of the two taxi-drivers in an eastern city who were positively identified by eye-witnesses as the perpetrators of a brutal crime, and were exonerated only after the real criminals confessed. In contrast to the notorious unreliability of subjective evidence, blood grouping evidence is purely objective and always reproducible.

3.35 Disputed Paternity

3.351 Blood-Grouping Tests

In applying blood grouping in bastardy proceedings, we have to consider the genetic compositions of the putative father and the known mother. We should also remember that at birth the agglutinogens are accompanied by the complementary agglutinins in only about one-half the infants. Since, however, the agglutinogens are already formed at birth, the agglutinins seldom create any uncertainty, even in tests done soon after birth. The important thing to remember here is the possibly greater weakness of factors like A_2 in the very young.

The kinds of offspring from matings that involve only groups O and AB are relatively simple to predict, since each of these groups (phenotypes) has but one genotypic formula (OO or AB). But if either parent is an A or a B, there are two genetic formulas possible for each, which we are not able to distinguish (without study of ancestors and known progeny, or in some cases by special serological investigation) (sec. 2.511), so the problem of prediction becomes somewhat more complicated.

Sample diagrams were given in table 25, showing the kinds and proportions of the children's blood groups engendered by homozygous × homozygous (AA × AA, example I), heterozygous × heterozygous (AO × BO, example III), and homozygous × heterozygous (AA × AO, example II) matings. By determining the kinds of offspring that can or can not result from all the 21 possible (genotypic) kinds of mating, we construct table 26. Omitting impossible mother-child combinations, those offspring that can not result from a mating of the accused man and the woman in question indicate his non-paternity, and are summarized in table 29.

This table gives the combinations of putative father, identified mother,

and identified child which prove that the man is innocent, and that some other must have fathered that particular child.

Some may find it helpful to have a table giving the conclusions which may be drawn about the father of a child of given blood group, born of a mother of known blood group. This can be deduced from table 26, and is presented in table 30.

The possibilities of exclusion given in these tables are those resulting from investigations of the three persons in question, but if relatives are investigated also, additional exclusions are sometimes possible. In some cases these may not be definite, but can be used as indications. For

TABLE 29

COMBINATIONS ALLOWING THE MAN TO ESTABLISH NON-PATERNITY, OMITTING INSTANCES OF IMPOSSIBLE MOTHER-CHILD COMBINATIONS

(Condensed from table 26)

Putative father	Known mother	Known child
O	O	A, B
O	A	B, AB
O	B	A, AB
O	AB	AB
A	O	B
A	A	B, AB
B	O	A
B	B	A, AB
AB	O	O
AB	A	O
AB	B	O

further details see Koller (13). For example, a man of group A, whose parents both belong to group AB, has a genetic formula AA, and therefore can not be the father of children of groups O and B; an analogous statement may be made for a man of group B from the same combination of parents.

3.352 Direct Reaction Between the Blood of Child and Putative Parents

In testing the blood group compatibility of a given case, we are not limited to determinations using known test sera, since A and B are inherited as dominants. It is possible to make an agglutination test, using the blood cells of the child and the serum of the parents, and to draw the same conclusions regarding paternity as from blood group determination

(22). This does not apply, however, to exclusions based on the combination, Child O, father AB, and vice versa.

The adjoining scheme shows the possible behavior of the child's cells with the sera of the parents:

		Agglutination of child's cells by	
		Mother's serum	Father's serum
Possibility	1	−	−
	2	−	+
	3	+	−
	4	+	+

TABLE 30

CONCLUSIONS POSSIBLE REGARDING BLOOD GROUP OF REAL FATHER OF CHILD FROM MOTHER OF KNOWN GROUP

I

Group of child	Mother of group			
	O	A	B	AB
	Possible groups for father			
O	O, A, B	O, A, B	O, A, B	Impossible
A	A, AB	—*	A, AB	—*
B	B, AB	B, AB	—*	—*
AB	Impossible	B, AB	A, AB	A, B, AB

II

Group of child	Mother of group			
	O	A	B	AB
	Impossible groups for father			
O	AB	AB	AB	Impossible
A	O, B	—*	O, B	—*
B	O, A	O, A	—*	—*
AB	Impossible	O, A	O, B	O

* No conclusion possible.

If any of the reactions 1, 2, or 3 is obtained, then the child could be the result of the supposed combination; but if the corpuscles of a child are agglutinated by the serum of both supposed parents (case 4), then the origin of the child from this mating is excluded, except for the possibility that it may be a child of group AB from parents A and B. This possibility may be investigated without blood group determination by mixing the serum of the father with cells of the mother, and vice versa. If both mixtures give positive agglutination, then we have the combination A × B and a

child of group AB. In this case, the child could have originated from the combination in question. But if even one of the reactions between the parents is negative, then the descent of the child from them may be denied. Thus questions of parentage could, if necessary, be tested without the aid of standard reagents.

The tests may be carried out in this order: first, the cells of the child are tested with the *serum of the mother*. If there is no agglutination, the child could be descended from the parental combination in question, and the blood group investigation will not be conclusive, so that further tests are unnecessary. If the mother's serum agglutinates the child's cells, then they must be tested also with the *father's serum*. Then there are two possibilities: (1) the father's serum *does not agglutinate* the child's cells. In this case, the child could come from the combination in question. The father's blood "corresponds to expectation". (2) The father's serum agglutinates the child's cells. In this case, the bloods of the father and mother are tested against each other as described above. If the sera of father and mother agglutinate the opposite corpuscles, the parents correspond to expectation. If one of the two tests between the blood of the father and mother is negative, the child cannot have arisen from this combination.

3.353 Application of the Subgroups of A and AB

A theory of the inheritance of the subgroups of A and of AB has been proposed by Thomsen, Friedenreich, and Worsaae (24). They postulate that instead of the three allelomorphic genes O, A, and B of Bernstein's theory, there exist four allelomorphic genes, O, A_1, A_2, B. Genes A_1, A_2, and B are supposed to be dominant over O, and A_1 dominant over A_2. (The theory could be extended to cover subgroups of B by postulating two B_1 and B_2 genes instead of B).

This theory in no way contradicts the fundamental principles of Bernstein's theory, but merely elaborates it; if no distinction is made between the subgroups, it reduces to Bernstein's theory.

If it could be taken for granted that this theory of heredity of the subgroups were entirely accurate, table 31 could be applied in cases of disputed paternity. Since, however, the exceptions to this theory are apparently too many to make illegitimacy a reasonable explanation (25), it is perhaps better to wait until the theory is better established. In any case, Wiener has calculated that the chances of disproving paternity by this means amounts only to 3 per cent, so the total efficiency (sec. 3.357) of blood grouping in this connection would not be greatly increased by use of the subgroups. The technic of differentiating the subgroups is discussed in sec. 2.52.

3.354 The M and N Factors

3.3541 Principle. Since the inheritance of the M and N types, as proposed by Landsteiner and Levine (15), has been proved by numerous subsequent studies to be correct, these factors also can be applied to questions involving disputed relationship. After our detailed discussion of the heredity of the A, B groups, it will not be necessary to go into the heredity of M and N in so much detail.

3.3542 Heredity. The factors M and N are inherited exactly like A and B, except that *an individual always has one or the other or both, never*

TABLE 31

HEREDITY OF THE SUBGROUPS, ACCORDING TO THE THEORY OF THOMSEN, FRIEDENREICH, AND WORSAAE (24)

Mating	Children possible	Children excluded by this theory in addition to those given in tables 26 and 29
A_2 x O	O, A_2	A_1
A_2 x A_2	O, A_2	A_1
A_2 x B	O, A_2, B, A_2B	A_1, A_1B
A_2B x O	A_2, B	A_1
A_2B x A_2	A_2, B, A_2B	A_1, A_1B
A_2B x B	A_2, B, A_2B	A_1, A_1B
A_2B x A_2B	A_2, B, A_2B	A_1, A_1B
A_1B x O	A_1, B	A_2
A_1B x A_1	A_1, B, A_2B, A_1B	A_2
A_1B x A_2	A_1, B, A_2B	A_1B, A_2
A_1B x A_2B	A_1, B, A_2B, A_1B	A_2
A_1B x B	A_1, B, A_1B	A_2, A_2B
A_1B x A_1B	A_1, B, A_1B	A_2, A_2B
A_1 x O	O, A_1, A_2	
A_1 x A_1	O, A_1, A_2	
A_1 x A_2	O, A_1, A_2	
A_1 x B	O, A_1, A_2, B, A_1B, A_2B	
A_1 x A_2B	A_1, A_2, B, A_1B, A_2B	

neither. Not a single blood has been found having neither M nor N. So the possible genetic formulas are MM, MN, and NN, and, using the principles previously exemplified, the reader will readily be able to verify table 32. These groups are established during foetal life.

As is true with the A, B groups, in the study of actual families, we must be prepared for some apparent exceptions. The situation is shown in table 33, from (4). The numbers in parentheses represent exceptions to the theory of Landsteiner and Levine. The table is made up of data compiled by Mustakallio (18) plus the data of Matta (16); it shows that in studies on the inheritance of M and N, there have been found 11 discrepant children

out of 5,281, or an average of 2.1 per 1000 children. Since the M, N types will detect about one sixth of the illegitimacies (13, 21, 28), this leads to the supposition that on the whole about 12 out of 1000 children in this series were illegitimate, a figure in startling agreement with that calculated (v. supra) from the exceptions to the Bernstein theory. The two studies thus supplement each other, and together effectively remove any doubt as to the essential correctness of the theories of inheritance of Bernstein, and of Landsteiner and Levine.

TABLE 32

INHERITANCE OF THE M, N TYPES ACCORDING TO THE THEORY OF LANDSTEINER AND LEVINE

Mating	Per cent of children of types			Mating	Per cent of children of types		
	M	MN	N		M	MN	N
M x M	100	0	0	M x MN	50	50	0
N x N	0	0	100	N x MN	0	50	50
M x N	0	100	0	MN x MN	25	50	25

TABLE 33

SUMMARY OF STUDIES ON THE INHERITANCE OF THE M, N TYPES

Mating	Number of families	Children of type		
		M	MN	N
M x M	175	497	(2)	0
N x N	98	0	0	294
M x N	196	(1)	555	(2)
M x MN	503	718	747	(4)
N x MN	375	(2)	541	584
MN x MN	491	302	734	298
Total.....	1839	1520	2579	1182
		5281		

The attribution of the observed "exceptions" to illegitimacy is rendered all the more certain by Wiener's observation (25) that in all the exceptions of the type; parent M, child N, or parent M, child N, where the discrepancy exists irrespective of the type of the other parent, it is always the father, never the mother, who is implicated. If these were real exceptions to the laws of heredity, the mother should be implicated just as often as the father.

3.3543 Application. The application of the laws of inheritance of M and N to cases of disputed paternity is obvious from table 34. The

possibilities of exclusion of paternity (or maternity) may be divided into two classes: (1) The blood factors M or N of a child must have been present in one of the parents. (2) A pure type N or M can not be present among the parents of a child who belongs to the opposite pure type M or N.

To test the first possibility, both of the putative parents must be tested. To test the second, one may investigate first the child and the doubtful parent. In some cases, paternity (or maternity) may be excluded without testing the second parent. It may be said further of a child of one of the pure types (M or N) that even the grandfather and grandmother could not both have belonged simultaneously to the opposite pure type (N or M).

Sometimes it is also helpful to test brothers and sisters. If two sibs belong to types M and N respectively, then both parents must belong to type MN. But in general we must be contented with tests on the child, the mother, and the putative father.

Since only three types exist, the individual possibilities may be outlined more simply than with the four blood groups. The following table shows

TABLE 34

EXCLUSION OF PATERNITY ON THE BASIS OF CHARACTERISTICS M AND N

Child	Mother	Father not possible
M	M or MN	N
N	N or MN	M
MN	M	M
MN	N	N

under what circumstances a child could not be descended from a certain man (table 34).

3.3544 Tests Without Absorbed Anti-M and -N Testing Fluids. If crude immune sera anti-M and anti-N are available, paternity can sometimes be tested without the necessity of preparing absorbed testing fluids although, needless to say, the latter is the preferable procedure.

Paternity may be excluded if characteristic M or N is present in the child and is absent in the putative parents. The test is carried out according to the following scheme which is drawn up for the characteristic M; exactly the same relations hold for N.

Immune serum anti-M absorbed with cells of	Test of absorbed serum against cells of		
	Child	Mother	Putative father
Mother	+	−	−
Putative father	+	−	−

If the experiment comes out as shown here, paternity is excluded, for the child is shown to possess the characteristic M which is absent in both the putative parents.

Paternity is excluded if the child and the putative father belong to the opposite pure types (child M, putative father N, or vice versa). This could be demonstrated by showing that one of the bloods (child or putative father) removed all agglutinins from an anti-M serum, say, but left anti-N when used for absorbing a crude anti-N, whereas the other blood had the opposite effect. In the case of a child of type M and a putative father of type N, the following results would be obtained:

Serum	Absorbed with cells of	Test of absorbed serum against cells of	
		Child	Putative father
1. anti-M	Putative father	+	−
2. anti-N	Child	−	+

The absorbed immune sera are seen to show cross-agglutination. Corresponding results would be obtained in the case of a child of type N and father of type M, only the sera being interchanged.

3.355 Procedure in Paternity Cases

3.3551 Identification. In all forensic blood investigations, care must be taken that the blood samples actually come from the persons they are supposed to. Without special precautions, the expert runs the risk of occasional substitution of other people. The identity of the persons appearing for the test must be definitely established at the time the blood is taken, for subsequent doubt as to the identity of one of the persons involved may delay a decision by several months, and a subsequent check-up is not always possible.

The identity of the people tested is most easily established by having them identify each other, if they appear at the same time. Otherwise, identification by an employee or other trustworthy person will serve. Photographs are useful, for they may be presented in court and will facilitate subsequent investigations. Adults should also be required to give their signature, which may be written in the notebook where the findings of the test are going to be recorded.

It is more difficult to check the identity of infants, but since as a rule no one will have any interest in exchanging them, one may usually be satisfied with the statements of the persons accompanying them. Especial care is necessary in investigations in which another expert has declared the mother's statement as to the child's paternity incorrect.

The most certain method of identification is finger prints, or in the case of infants, hand or foot prints.

3.3552 Taking the Blood. From adults blood may be taken by venipuncture as for the Wassermann reaction. This method is most convenient for serological investigation because sufficient cells, as well as

serum, are then available. About 0.5 cc of blood is generally sufficient. However, venipuncture is not necessary. It is possible to get along with blood taken from the lobe of the ear or from a finger. If blood is taken in this way, even the most apprehensive person can be convinced of the slightness of the inconvenience required of him, and will have no reason to refuse to give blood because of any fancied discomfort. It may be well to take two samples, in different tubes, from each person. With a little practice, it is not difficult to obtain 0.5 cc. of blood from an ear or a finger.

It is not worth while to attempt to obtain serum from a new-born infant, for his own serum agglutinins are as a rule not formed yet. Blood sufficient for cells can be taken from the big toe, or the heel. From older children, it is best taken from the ear lobes.

3.3553 Transport of Blood Samples. The same rules hold as for sending samples for the Widal and Wassermann tests. Blood dilutions are not suitable for transportation, but must be worked on at once (compare 2.232). It is especially important to take blood with sterile precautions and to use sterile containers. For small amounts (taken from the ear lobe) one may use the little tubes intended for the Widal reaction, sterile capillaries or Neisser U-tubes or Wright capsules. The ends of the capillaries should be sealed airtight in the flame or with sealing wax. Mixture with foreign material such as alcohol should be avoided. Preservatives are not in general advisable, although blood mixed with Rous and Turner solution (sec. 2.1) has been shipped successfully, especially when kept cold.

The samples should be clearly marked, on the wrapper and on the container itself. They should be sent by air mail when this is possible.

3.3554 Making the Tests. Tests in cases of disputed paternity have the advantange over other forensic blood investigations that fresh blood in sufficient amounts can nearly always be obtained. Therefore the test tube method (secs. 2.251, 2.4412) will be used. The general technic is as previously described.

By testing each blood with several sera of each kind, and by including proper controls, the possibility of error is excluded. Each blood is tested with strong human sera of groups A and B, and also, if at all possible, with absorbed immune sera anti-B and anti-A. At least two, preferably three, different anti-M and anti-N sera are used. The sera from the persons being tested are reacted with A and B cells which are known to be sensitive. In each set-up, known bloods of groups O, A (preferably including both subgroups A_1 and A_2), and B; and types M, MN, and N are included. The MN is necessary because corpuscles of the mixed type are in general less sensitive.

Those who have become familiar with the very sharp results which

may be obtained with strong immune sera after skillful absorption may be inclined to consider some of the above controls superfluous; nevertheless, they should never be omitted in any medico-legal investigation.

If paternity appears to be excluded, the tests are all repeated, with different reagents, using the duplicate blood samples (above). The sera used must be known to be suitable in respect to (1), *strength:* an absorbed immune serum is strong enough for forensic purposes when the amount of serum dilution which is used contains at least ten agglutinating doses; that is, when $\frac{1}{10}$ of the amount going to be used in the test will still give strong agglutination. (2), *Specificity:* an immune serum is specific if after absorption, in the dilution used, it contains no trace of any agglutinin other than the one desired. It must not be assumed that because blood of group A or group B, say, was not used for the immunization, therefore anti-B or anti-A must be absent. The same is true for anti-P, and anti-M or anti-N.

In addition to watching for antibodies against known blood characteristics, the investigator should be on his guard against possible antibodies to hitherto unknown, new type characteristics. Before using any new immune serum, rather extensive tests of specificity must be undertaken.

3.3555 Records. The results of the paternity test should be entered in a notebook or on a protocol sheet similar to the adjoining model (table 35).

To provide for the possibility that subsequent rechecks may be needed, the origin of the test bloods should be indicated precisely. If special lists of test bloods are kept, then it is sufficient to give the numbers from this list.

The results of the tests may be preserved as documentary proof by photography, or more simply by pouring the contents of the individual test tubes on filter paper to dry (sec. 3.37), (see figure 31).

In the case shown in table 35, (the names are fictitious), an exclusion of paternity is shown by the A, B system and by the M, N system.

3.3556 Reports. When a written report is requested (as is usual), we have found it convenient to use a mimeographed form similar to that shown in figure 30. If the test is conclusive for non-paternity, and the evidence is later introduced into court, the more detailed original protocol may be produced.

3.356 Conclusions in Paternity Cases

If the M, N, types are incompatible with the law of inheritance of Landsteiner and Levine, or if the blood groups are incompatible with the law of von Dungern and Hirszfeld (A or B present in child when absent in

TABLE 35

PROTOCOL OF TESTS IN CASE OF DISPUTED PATERNITY

I. Blood group determination

A. Test of cells with known serum (3 test sera A (anti-B), 3 test sera B (anti-A))

Cells from	Name	First name	Test sera						Blood group
			A (anti-B)			B (anti-A)			
			267	273	[301	250	261	[267	
Child	Walker	Martha	−	−	−	+	+	+	A
Mother	Walker	Mary	−	−	−	−	−	−	O
Putative father	Lamar	Francis	−	−	−	−	−	−	O
		number							
Control cells	O	300	−	−	−	−	−	−	
	A_1	267	−	−	−	+	+	+	
	A_2	268	−	−	−	+	+	+	
	B	290	+	+	+	−	−	−	

B. Test of sera with known cells (4 samples of A cells, 4 of B cells)

Sera of	Name	First name	Test cells A				Test cells B				Blood group
			238	275	[301	[306	247	250	[261	[298	
Child	Walker	Martha	−	−	−	−	+	+	+	+	A
Mother	Walker	Mary	+	+	+	+	+	+	+	+	O
Putative father	Lamar	Francis	+	+	+	+	+	+	+	+	O
Control serum		number									
[O (anti-AB)		302	+	+	+	+	+	+	+	+	
A (anti-B)		301	−	−	−	−	+	+	+	+	
B (anti-A)		261	+	+	+	+	−	−	−	−	
[AB(o)		307	−	−	−	−	−	−	−	−	

II. Determination of blood type

Cells from	Name	First name	Anti-sera						Blood type
			anti-M			anti-N			
			710	711	[712	633	634	[641	
Child	Walker	Martha	+	+	+	+	+	+	MN
Mother	Walker	Mary	+	+	+	−	−	−	M
Putative father	Lamar	Francis	+	+	+	−	−	−	M
Control cells									
M			+	+	+	−	−	−	
MN			+	+	+	+	+	+	
N			−	−	−	+	+	+	

The symbol [indicates tests which may be omitted.

Date_____

To_____

The results of Blood Grouping Tests made by at 78 E. Concord Street, Boston, on the above date are as follows:

Name	Blood Cell Composition
Mother	
Child	
Alleged Putative Father	

When mother, child and alleged putative father have respective blood grouping types listed above:

*A: it is impossible to draw any conclusions as to whether or not the alleged putative father is actually the natural father of the child in question. These results should in no wise be considered as incriminating evidence as to paternity. In such an event the Blood Grouping Tests are said to be inconclusive.

B: the alleged putative father cannot be the natural father of the child in question, because the child inherited the blood character
which was present neither in the mother nor in the alleged putative father, and hence must have been present in the natural father from whom inherited it.

*Strike out paragraph not applicable.

Assoc. Prof. of Biochemistry
Boston University
School of Medicine

Fig. 30. Typical form for report of results of non-paternity test. (*Adapted from a form devised by Dr. Clyde Keeler.*)

both parents), it may be stated categorically that the accused man is not the father. If the groups are incompatible with the second law of Bernstein (child of group AB from O father, or O child from AB father), the probability of the innocence of the accused is still practically overwhelming. However, if the AB belongs to subgroup A_2B (26), it is perhaps safest not to state that the proof is absolute, in view of the case of Haselhorst and Lauer (10) discussed above. Wiener points out that this is a conservative attitude, but in any case about $\frac{5}{6}$ of the exclusions possible with the A, B groups are made by the first law, and only about $\frac{1}{6}$ by the slightly less certain second law.

3.357 Chances of Proving Non-paternity

It will be understood without detailed explanation of calculations that, knowing the percentage distribution of the blood groups among the local

Table 36

Probabilities of Proving Non-Paternity When Blood Group of Wrongfully Accused Man is Known (12)

Group	Approximate per cent in the United States	Probabilities
O	45	1/5*
A	42	1/17
B	10	1/7
AB	3	1/2
(If unknown		1/7)

* 1/5 = 1 in 5 = 20 per cent.

population, we can calculate the chances that if we determine the blood groups of putative father, known mother, and infant, one of the combinations in table 29 will be found, and thus establish non-paternity. It is obvious that this will not always happen, for the real father may well be in the same group as the accused; for example, almost half of our population belong in group O. If we know the group of the accused, we can calculate the chances that the groups of the mother and offspring will be such that non-paternity can be proven. These chances differ with each group. They have been calculated for special cases, and the results for the white population of the United States (20, 11) are shown in table 36.

It appears (see "Unknown" in table 36) that, on the average, using only the O, A, B, AB determinations, the chances are 6 to 1 against an innocent man's being thus exonerated by recourse to these groups alone; he has but one chance in 7.

General formulas have been derived (31, 28, 13) which enable the

chances of establishing non-paternity to be computed at once for any population if the blood group frequencies are known. Such formulas are usually stated in terms of the frequencies of the genes O, A, and B.

These frequencies are easily calculated on the basis of the Bernstein theory (cf. sec. 3.65142). Let p, q, and r, represent the frequencies in the population of the genes for A, B, and O respectively. Then $p + q + r = 1$, and $(p + q + r)^2$ will give all possible combinations of these genes two at a time (genotypes). Allowing for the fact that the heterozygotes are routinely indistinguishable from the homozygotes in the case of groups A and B, we obtain: $p^2 + 2pr = \bar{A}$, $q^2 + 2qr = B$, $2pq = \bar{A}\bar{B}$, $r^2 = \bar{O}$. The gene frequencies are easily obtained by solving these equations for p, q, and r, when $\bar{O}$, $\bar{A}$, $\bar{B}$, and $\bar{A}\bar{B}$ represent the frequencies (per cent/100) of these groups; or it is easily shown that: $p = 1 - \sqrt{\bar{O} + \bar{B}}$, $q = 1 - \sqrt{\bar{O} + \bar{A}}$, $r = \sqrt{\bar{O}}$.

Table 37

General Chances of Establishing Non-Paternity by Blood Group Determinations, for Innocent Men in Various Groups

$$P/O = r^2(p + q) + 2\,pq(1 + r)$$
$$P/A = q(p + r)^2$$
$$P/B = p(q + r)^2$$
$$P/AB = r^2$$
$$P/O, A, B, AB = p(q + r)^4 + q(p + r)^4 + pqr^2(p + q) + 2\,pqr^2$$

(The symbol P/O means the probability of thus establishing non-paternity for a man in group O.)

Table 38

General Formulas for Chances of Establishing Non-Paternity for Innocent Men of Various M, N Types

Type	Probability	Type	Probability
M	$n(1 - mn)$	N	$m\ (1 - mn)$
MN	0	In general	$mn(1 - mn)$

The general formulas, giving the chances of establishing non-paternity in terms of gene frequencies, are given in table 37.

The chances of excluding paternity by M, N tests can be calculated in the same way. It is found that use of the M, N types provides about the same net chance of establishing non-paternity as do the classical blood groups. General formulas for these chances have also been derived (28,

21, 13). They are given in table 38. The symbols m and n represent the frequencies of the genes for M and N respectively, best estimated by

$$m = \overline{M} + \tfrac{1}{2}\overline{MN}$$
$$n = \overline{N} + \tfrac{1}{2}\overline{MN}$$

where $\overline{M}$, $\overline{MN}$, and $\overline{N}$ stand for frequencies (per cent/100) of these types in the population.

It will readily be understood that if the A, B and M, N tests are applied together, the total chances of establishing non-paternity are approximately

TABLE 39

CHANCES OF ESTABLISHING NON-PATERNITY BY USE OF A, B, M, AND N FACTORS (AFTER WIENER)

Putative father of type	Probabilities	
	Per cent	About
OM	50.71	1/2
ON	56.00	3/5
OMN	25.18	1/4
AM	40.37	2/5
AN	46.67	1/2
AMN	9.05	1/11
BM	43.59	2/5
BN	49.56	1/2
BMN	14.38	1/7
ABM	58.36	3/5
ABN	62.77	3/5
ABMN	36.81	2/5
Unknown	33.07	1/3

double those for either system alone. The numerical values for a typical population (New York City) as calculated by Wiener, are shown in table 39. It will be observed that in this population the chances in general amount to one in three.

3.3571 Computation of the Chances of Establishing Non-paternity

Table 29 summarizes all the instances in which the designated child of the known mother could not be the offspring of the alleged father. The problem is to calculate the probability of the occurrence of these combinations.

First we must determine the probability that an individual taken at random will be in a given group. This is given directly by the percentage of the various groups among the population with which we may be con-

cerned. The figures for the United States are given in table 36. Thus the chance that a man (or woman) will be in group O are $\frac{45}{100}$; but in group AB, only $\frac{3}{100}$. These frequency distributions are independent of sex. Next we must remember that the probability that a child will be in a given group depends upon the genotypic constitutions of its parents. For example, an O child may result from an AO $\times$ AO mating, but not from AA $\times$ AO, or AB $\times$ OO. This necessitates a calculation of the frequency of each genotype so that we may then compute, as we must, the group distribution of children that are born of women in each group.

Genotype frequencies are obtained as follows: there are three genotypic factors, A, B, and O. Let p = frequency of factor A; q = frequency of factor B, and r of factor O; then $p + q + r = 1$. Random mating gives all the possible combinations, represented by $(p + q + r)^2$, and their sum should equal unity. Expanding this algebraic expression we obtain

$$p^2 + 2\,pr + q^2 + 2\,qr + 2\,pq + r^2$$

and these symbols represent the frequencies of the 6 genotypes

AA AO BB BO AB OO

We can now derive formulas that enable us to express genotype frequencies in figures, by substituting in these formulas the frequencies (per cents divided by 100) of the groups already determined by testing a large enough sample of the population. The derivation follows:

$$r^2 = \bar{O} \tag{1}$$

$$2pr + p^2 = \bar{A} \tag{2}$$

$$2qr + q^2 = \bar{B} \tag{3}$$

So,

$$r = \sqrt{\bar{O}} \tag{4}$$

And, adding (1) and (2)

$$(r + p)^2 = \bar{O} + \bar{A}$$

$$r + p = \sqrt{\bar{O} + \bar{A}}$$

$$p = \sqrt{\bar{O} + \bar{A}} - \sqrt{\bar{O}} \tag{5}$$

In the same way, adding (1) and (3),

$$q = \sqrt{\bar{O} + \bar{B}} - \sqrt{\bar{O}} \tag{6}$$

Since there is but one genotype in group O and one in AB, no formula is needed to obtain their frequencies. Omitting a description of the simple process of substituting figures in the above formulas we may now tabulate (table 40) the frequency distribution of the genotypes, based on the approximate group frequencies in the United States.

The figure for group AB derived by applying the formula sometimes deviates slightly from that obtained by sampling the population, since p, q, and r are calculated from the frequencies of the other three groups. More exact statistical methods (sec. 3.65142) of estimating p, q, and r have been proposed, but are not needed for our purposes.

Having, now, the frequency distribution of the genotypes, it is possible to compute the group frequencies of the progeny of all the mating combinations. It will be evident that women in group O, mated with men in group A of genotype AO, (mating OO × AO) will bear, on the average,

TABLE 40

FREQUENCIES OF THE SIX GENOTYPES

Group	Genotype	Frequency		
O	OO		0.450	(r^2)
A	AA	0.42	0.068	(p^2)
	AO		0.352	(2pr)
B	BB	0.099+	0.005	(q^2)
	BO		0.094+	(2qr)
AB	AB		0.03	(2pq)

50% of group A and 50% of group O children (cf. diagrams in table 25). Since men of genotype AO constitute 35.2 per cent (table 40) of the male population, then 17.6 per cent of the children of women in group O will be in group A due to this kind of mating. The calculations for other mating combinations are similarly made, and all are combined in table 41.

Now we may proceed to the final computations. The probability that two or more independent events will happen together is given by the product of their individual probabilities. Selecting those instances permitting a man in group O to establish non-paternity (top section of table 29), and multiplying the individual frequencies (tables 40 and 41), we obtain the probabilities listed in table 41a. Inasmuch as the probability that, of two or more independent events, one or another will happen, is the sum of the probabilities of the individual events, we add the fractions of the right hand column of table 41a and obtain $\frac{2057}{10{,}000} \cong \frac{21}{100} \cong \frac{1}{5}$, which is the probability that a wrongfully accused man in group O will be able to establish his innocence by these tests.

The calculations are of the same type when the man is in group A, B, or AB.

If the putative father's group is not known, we can compute only the probability that, whichever group he proves to be in, he will be able to establish non-paternity. The chances that he is in group O are $\frac{45}{100}$ and if so, the probability that we can prove his innocence is $\frac{2057}{10,000}$. The

TABLE 41

APPROXIMATE FREQUENCIES (IN THE U. S. A.) OF THE VARIOUS GROUPS AMONG CHILDREN OF WOMEN (OR MEN) OF EACH OF THE FOUR GROUPS

Women	Children			
	O	A	B	AB
O	0.67	0.26	0.07	
A	0.28	0.65	0.03	0.04
B	0.35	0.10	0.42	0.13
AB		0.47	0.37	0.16

TABLE 41a

PROBABILITIES OF ESTABLISHING NON-PATERNITY WHEN THE WRONGFULLY ACCUSED MAN IS IN GROUP O

Mother's group	Child's group	Probability (product)
O 45/100	A 26/100	1170/10,000
O 45/100	B 7/100	315/10,000
A 42/100	B 3/100	126/10,000
A 42/100	AB 4/100	168/10,000
B 10/100	A 10/100	100/10,000
B 10/100	AB 13/100	130/10,000
AB 3/100	AB 16/100	48/10,000
		2057/10,000

product is $\frac{92,565}{1,000,000}$. Dealing similarly with the other cases, and adding the results, we get $\frac{141,800}{1,000,000} = \frac{14}{100} = \frac{1}{7}$.

The derivation of the general formulas, which are applicable directly to the group frequencies of any population, would have to be explained more abstractly and would be in general more difficult to understand.

Mathematical readers who have followed the above exposition will readily conceive of the appropriate generalizations.

The chances of proving non-paternity by use of the M, N types can be calculated in a similar way.

3.358 Results in Actual Practice

Blood tests have been used in the European courts in paternity cases since 1924, having been introduced first in Germany through the efforts of Schiff. The first case was in Berlin. The first legal case in which the M, N system was used occurred in Potsdam in 1931. As early as 1929 Schiff was able to compile statistics on 6665 tests in various parts of Europe; his own results for Germany are presented in table 42.

It will be noted that using both systems, 14 per cent, or about 1 in 7, of the accused men were proved innocent. It has been calculated (sec. 3.357) that it should be possible to establish innocence in about one-third of the cases, provided all the accused men were really innocent. We may therefore conclude that somewhat over half of the men tested by Schiff were really guilty. Similarly it has been found (19) that in New York City 8.72 per cent of exclusions are obtained, leading to the conclusion that out of 100 men accused of this offense in New York, in the long run 26 are innocent and 74 guilty. Schatkin and Levine point out that without the blood tests, several or perhaps all of the innocent men would have been adjudged the father and would today be supporting children not their own. "These figures are eloquent of the value of the blood tests."

TABLE 42

ACTUAL RESULTS OF SCHIFF'S TESTS FOR NON-PATERNITY IN GERMANY, 1924–1934 (RESULTS FOR YEAR 1929 NOT AVAILABLE)

Examination for	Chances of excluding an innocent man (per cent)	Number of court cases		
		Tested	Exclusions of paternity in	
			number	per cent
O, A, B	17.6	1822	140	7.7
M, N	18.6	1269	109	8.6
Both systems	33.0	1129	160	14.2

The blood tests were not used extensively in the courts of the United States before 1934. In 1934 the tests were ordered by a higher court for the first time in this country in a case tested by Wiener (1). Unfortunately the decision rendered in this case was reversed by the Appellate Division on the grounds that there was no statutory authority for the order for the blood test. Such authority was soon provided by the passage of laws

dealing with the subject (sec. 3.3821), sponsored by Dr. A. S. Wiener. Three other states (Wisconsin, Ohio, New Jersey) have since passed similar laws. The laws in both Wisconsin and New Jersey were written and sponsored by Dr. Philip Levine.

3.36 Application to Other Cases Involving Relationship

From tables 25 and 26 it will be seen how blood tests can in some cases render assistance in other affiliation cases. Non-maternity can be proved in a small percentage of cases, but of course this is rarely applied.

Interchange of infants, as in hospitals, can be detected, using both systems, in about 66 per cent of cases, and blood tests have been applied successfully (18a, 25) in such mixups.

One of the best-known cases of this sort is that of "Mrs. W" who found, on returning home from the hospital, that her infant had on its back a piece of adhesive tape bearing the name "B". The baby at the "B" house was found to have a label with the name "W". The bloods of the six persons involved were tested, with the following results:

	Group		Group
Mr. B	AB	Mr. W	O
Mrs. B	O	Mrs. W	O
Baby "W"	O	Baby "B"	A

It is clear that interchange is doubly proved, for in neither case could the infant brought home be the right one. Mr. B, a group AB, could not have an O child, and Mr. and Mrs. W., both O, could not have an infant of group A. Clearly the children had accidently been interchanged before the mothers went home, and the labels they bore were correct. The infants in this case were correctly allocated even without recourse to the M, N types.

3.37 Preservation of Results of Tests

Sometimes, as in court cases, it is helpful to have something more concrete to exhibit than a mere entry in a notebook. For this purpose, and others, it is convenient to preserve the actual positive and negative tests. This may be done very simply by pouring the contents of each tube onto a separate area of a not-too-white filter paper. A positive test leaves a single dark red speck or a group of little specks, while a negative test leaves on the paper merely a diffuse, yellowish area. The red of course soon turns brown, but the characteristic difference between positive and negative will remain clear for many years. The paper can subsequently be protected by covering it with cellophane. It is easy to photograph (see figure 31).

For photographic evidence ordinary micro-photographs, slightly enlarged, may be used. Without a camera, an objective record may be ob-

tained if photographic paper is placed immediately under the slide and illuminated from above. The resulting silhouettes reproduce well the macroscopically visible difference between negative and positive reactions.

Reactions cannot be preserved in the test tube for a long time. Even during classroom demonstration the clumps may gradually break up as a result of agitation. This may be avoided if the circumambient fluid is made solid by the addition of agar-agar. 2 or 3% agar is melted in a test tube; just before it re-solidifies, the (agglutinated or unagglutinated)

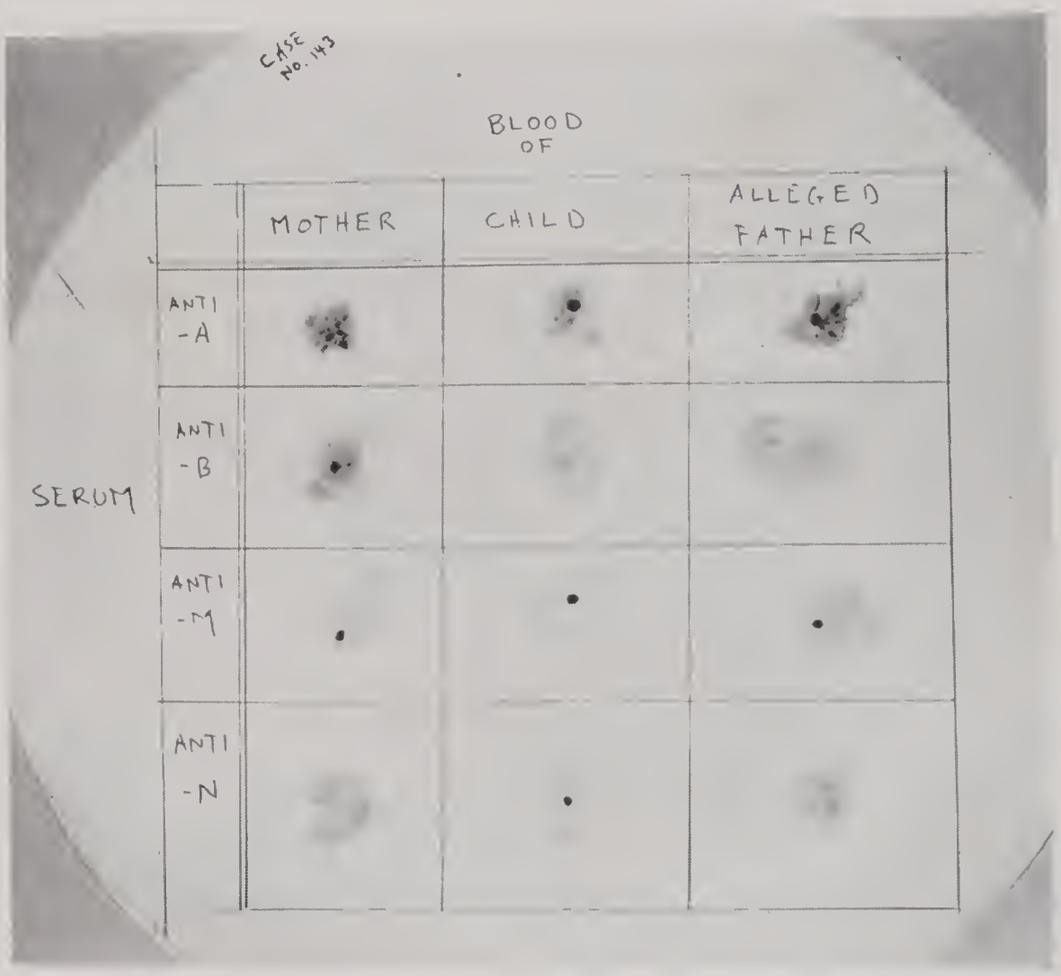

Fig. 31. Preservation of agglutination tests by drying on filter paper

corpuscles are added in suitable amounts and evenly distributed by careful mixing.

3.38 Laws Relating to the Use of Blood Grouping Tests

3.381 In Europe

The situation in Europe (at least before the second World War) is summarized by Christiaens (5).

3.382 In the United States

New York enacted a statute, giving the courts power to order blood tests, on March 22, 1935. Similar laws were passed in Wisconsin on August 9, 1935, in Ohio on June 21, 1939, and in New Jersey on July 18, 1939.

3.3821 New York Law. The New York law is found in the Inferior Criminal Courts Act, Section 67, Subdivision 1a. It provides:
"The court, on motion of the defendant, shall order the mother, her child and the defendant, to submit to one or more blood-grouping tests by a duly qualified physician to determine whether or not the defendant can be excluded as being the father of the child, and the results of such test may be received in evidence but only in cases where definite exclusion is established."

3.3822 New Jersey Law.

"Assembly, no. 61.
"State of New Jersey.
"An Act concerning evidence and witnesses, and supplementing subtitle eleven, of Title 2, of the Revised Statutes.

"Be it enacted by the Senate and General Assembly of the State of New Jersey:

"Whenever it is relevant to the case of the prosecution or the defense in a proceeding involving parentage of a child, the trial court, by order, may direct that the mother, her child, and the defendant submit to one or more blood grouping tests to determine whether or not the defendant can be excluded from the probability of being the father of the child. The testimony of experts to the result of the test shall be receivable in evidence, but only in cases where definite exclusion of parentage of the defendant is indicated. The tests shall be made by duly qualified physicians, to be appointed by the court. Such experts shall be subject to cross examination by both parties after the court has caused them to disclose their findings to the court or to the court and jury. Whenever the court orders such blood grouping tests to be taken and one of the persons thus directed shall refuse to submit to such tests, such fact shall be disclosed upon the trial in the discretion of the court.

"Whenever it shall be relevant in a civil action to determine the parentage or the identity of any child or other person, the court, by order, may direct that any party to the action and the person whose parentage or identity is involved submit to one or more blood grouping tests, to be made by duly qualified physicians under such restrictions and directions as the court or judge shall deem proper. Whenever such test is ordered and made, the testimony of the experts to the results thereof, subject to cross examination as in section one, shall be receivable in evidence, but only in cases where definite exclusion is indicated. The order for such blood grouping tests also may direct that the testimony of such experts and of the persons so to be examined be taken by deposition. The court shall determine how and by whom the costs of such examination shall be paid.

"This act shall take effect immediately."

3.383 Comment

It will be noted that both laws provide that the tests are to be made by "duly qualified physicians". The Corporation Counsel of New York City, who is charged with the prosecution of paternity cases, communicated with the New York Academy of Medicine to obtain a list of physicians qualified for this work. It would be very unwise to appoint doctors taken at random to carry out this work, since the average practitioner does not possess the necessary experience, or even the materials, needed to carry out the tests.

On the other hand, as mentioned above, there are a number of blood grouping experts, geneticists and serologists, who do not possess medical degrees. We would recommend that in the future, when laws are passed in other states, the laws should be worded so that "duly qualified persons" can carry out the tests, as insistance on the medical degree will mean that many competent experts will be disqualified.*

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* In one of the four states mentioned above, the State Medical Society insisted on the *removal* of the phrase "or other qualified persons" from the proposed law!

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3.4 Forensic Applications of Blood Grouping, Including Blood Stains

Blood grouping tests carried out in paternity cases, which are, strictly, forensic applications too, have been treated above (sec. 3.3), and we shall restrict our discussion here to applications in connection with violent crime.

3.41 Group Determination of Blood Stains

3.411 General

Blood stains are frequently found at the scene of a violent crime. The importance of testing whether these are of human origin is generally recognized, and such tests are often carried out. Sometimes the group of the blood can also be determined, and in some cases this information may have an important bearing on the investigation of the crime. We may consider a simple hypothetical case: blood stains are found on the clothing or wearing apparel of a man accused of committing a murder which is known to involve blood-letting. He claims the blood is his own, resulting from a nosebleed, or an injury. The stain is tested, and found to be of a blood group different from that of the accused. The result can only be strongly to intensify already existing suspicion.

As in other special applications, it is essential that the tests be carried out only by qualified persons.

Determination of the group of stains is a reliable technic in the hands of experienced operators. We should like to emphasize that this does not mean that it can be carried out by unskilled individuals without practice, or even by those familiar with other aspects of blood grouping, but without special experience in this particular work. Probably few physicians would care to rely upon the results of uncontrolled Wassermann tests carried out on the bloods of their patients by a medical student fresh from the average course in immunology. In our opinion, the technic of testing dried blood stains is much more delicate and exacting than the serological test for syphilis, especially since a great deal more may depend on it, for an accused person's life might conceivably hang in the balance. No in-

dividual should venture to carry out these tests in a medico-legal case unless he has previously had experience in serology, and has *correctly* grouped at least 50 known blood stains, including all the major types. To do otherwise is to risk bringing discredit upon oneself and one's science.

3.412 Limitations

The grouping of stains, reliable as it is in experienced hands, has certain limitations which it would be foolish to ignore. In the first place, paradoxical as it may seem, the blood has actually a lower content of the group receptors than certain other tissues and fluids of the body; this, coupled with greater solubility, makes it actually easier to group seminal stains, and certain other materials, than to group blood stains (17, 32, 33).

Also, as has been pointed out by Hirszfeld and by Lewiński (13, 22), it is only in certain cases that a knowledge of the group of a blood stain can be of any use in a criminal investigation. If an alleged murderer and the victim were both of the same blood group, then a knowledge of the group of a stain on a handkerchief found in the accused's possession will not bear one way or the other on his guilt, if he should claim that stain to be from his own blood, as he will possibly do. The value of blood grouping here, as in paternity cases, is essentially *negative*; a positive result, although it might point suspicion, can never prove guilt, save in very exceptional cases of isolation.

A third disadvantage mentioned by various workers (5, 13, 22), is that the material on which the stain is found may often itself contain group receptors. An individual's underwear, if worn more than a few days, is almost sure to contain A and B receptors, if these are present in his blood. This information in itself may be of value in some cases, but it is evident that the presence of such receptors can in many cases make it useless to try to determine the group of the stained portion.

Lewiński (22) reported that out of 122 stains examined in the Warsaw Hygiene Institute, 17 were found, by the precipitin test, not to be of human origin. Of the remaining 105 stains only 12 contributed information of any value, since in the other 93 cases either the defendant and the victim belonged to the same group, or the unstained portions of the material gave the same reactions as the stain, or the stain had deteriorated to such an extent that it gave no specific reactions.

But in certain instances, proof of a difference in the group of a stain and that of a certain individual may dispel suspicion which was directed towards him, or give the lie to some statement made by him. In such cases, when a knowledge of the group of a blood stain would be helpful, methods similar to those given below (sec. 3.4132) should be applied. In the Medico-legal Laboratory in Cairo, for example, we were told that several blood stains a day were examined.

3.413 Procedure

3.4131 Preliminary. Before attempting to determine the group of a stain or other specimen of dried blood, it is necessary to ascertain first of all that it is blood (by the benzidine or tolidin test), then to determine if it is human blood. For the latter the precipitin test is used.

3.41311 Preparation of Precipitins. Precipitins suitable for differentiating human from other blood may be produced by injecting laked whole human blood rendered again isotonic by the addition of salt, or by injecting defibrinated whole blood, plasma, or serum. The material injected is called the antigen. The easiest material to procure is serum, and it seems on the whole as satisfactory as any.

The serum to be used for injection should be kept sterile, and the injections made with aseptic precautions, more to preserve the antigen from contamination than because there is any great danger that the injected animals will die of infection (assuming rabbits are used). If the human blood is taken aseptically into sterile containers and handled with bacteriological technic throughout, the serum may be used for the injection without further treatment, or after dilution 1:6 or 1:7. If there is doubt about its sterility, 'Merthiolate' (1:5,000), or possibly phenol (to 0.5%) may be added; or better, it may be sterilized by passing it through a Berkefeld filter.

The technic of injecting animals for the production of immune sera has been described above (sec. 2.322). Precipitating sera for forensic work are almost always prepared in rabbits, although chickens have been used.

There is great individual variation in the injection schedule of different workers, and no experiments have been done which are sufficiently elaborate to determine which is best. The animal may be injected intravenously once, than after 6 to 8 days intravenously again, then after 6 to 8 days intraabdominally, a trial bleeding being taken in 10 to 14 days (Uhlenhuth); or, the injections may be given intravenously the first three days of one week, followed by one intraabdominal and two intravenous injections the first three days of the following three weeks, followed by a trial bleeding one week after the last injection. Good antisera have been obtained by both of these, and by a variety of other methods.

If an animal is refractory and requires an unusually long course of injections, the antiserum obtained may be less specific than that from other animals.

No matter what the exact schedule followed, after a few injections have been given, later intravenous injections coming after the lapse of more than one or two days should be prefaced by an intraabdominal injection the preceding day, to avoid possible anaphylactic shock.

There is no apparent advantage in giving more than 1 or 2 cc. of a 1%

protein solution at each intravenous injection. Intraabdominal injections should be about three times as large. It is the practice of many to increase the size of the dose as immunization proceeds, but it is not certain that any better antisera are thus obtained.

The technic of bleeding rabbits is described above (sec. 2.3222). It is best to preserve the sera by sterilization by passage through a Berkefeld, or by addition of "Merthiolate", or both.

3.41312 Technic of the Precipitin Reaction. A precipitating serum, if mixed with the homologous (= from same species) antigen in the proper proportions, will produce a clouding of the originally clear fluids, usually followed by the formation of a flocculant precipitate. The reaction is more easily seen, and takes place over a wider range of concentrations, if it is carried out by placing one fluid (the antigen) over the other (the antiserum) in a suitable test tube. By this *interfacial technic* the reaction is visible as the formation of a white zone or plane at the junction of the two clear fluids. This is often called the "ring" test.

3.41313 Reagents. The reagents involved are two: antiserum and antigen. The preparation of both has been described above. Both should be crystal clear, though not necessarily colorless. If not clear, they should be filtered through a retentive filter paper, and if necessary centrifuged for a long time at high speed. The antiserum is used undiluted, or sometimes diluted 1:2 or 1:4. A number of different dilutions of the antigen should be tested. If successive dilutions of 1:10 are made of the stock antigen, giving dilutions of 1:10, 1:100, 1:1000, etc., six dilutions will be sufficient. The dilutions are made in isotonic (0.9%) salt solution.

3.41314 Apparatus. It is convenient to make the antigen dilutions in test tubes about 13 x 100 mm. (Wassermann tubes), using ordinary serological pipettes. The reaction may be carried out in test tubes of 9 x 110 mm., putting in 0.9 cc. of antigen dilution, and 0.1 cc. of antiserum (Uhlenhuth). Much material may be saved, and the reaction rendered easier to read, by using "micro"-tubes made from semicapillary tubing, about 35 mm. long, and 2.5 mm. internal diameter. In these there is placed about 0.04 cc. of antiserum and on top of this about 0.04 cc. of antigen dilution.

For the Uhlenhuth tubes special racks may be used in which the tubes hang from their lip and the bottom, in which the reaction takes place, is free. The little tubes recommended by us may be supported by sticking their lower end into plasticine in a small shallow container, such as the metal cover of a pill box (figure 32). Or special small racks of wire may be constructed, which have the advantage of leaving the bottom of the tube open to inspection, and of not soiling the outside of the tubes.

For use with the small tubes, capillary pipettes, made by drawing ordinary glass tubing to a point in a flame, and rubber bulbs (2 cc. capacity) will be needed. With a little practice, it is easy to transfer with these the desired amount of fluid into the little precipitin tubes.

3.41315 Determination of Titer of Antiserum. While there are more exact—but tedious—methods of determining the strength of antisera, it seems sufficient in this work to estimate it by determining the highest dilution of antigen which is precipitated by the antiserum. This is particularly appropriate here as we are interested in having a serum which will detect blood proteins, in very low concentration if necessary.

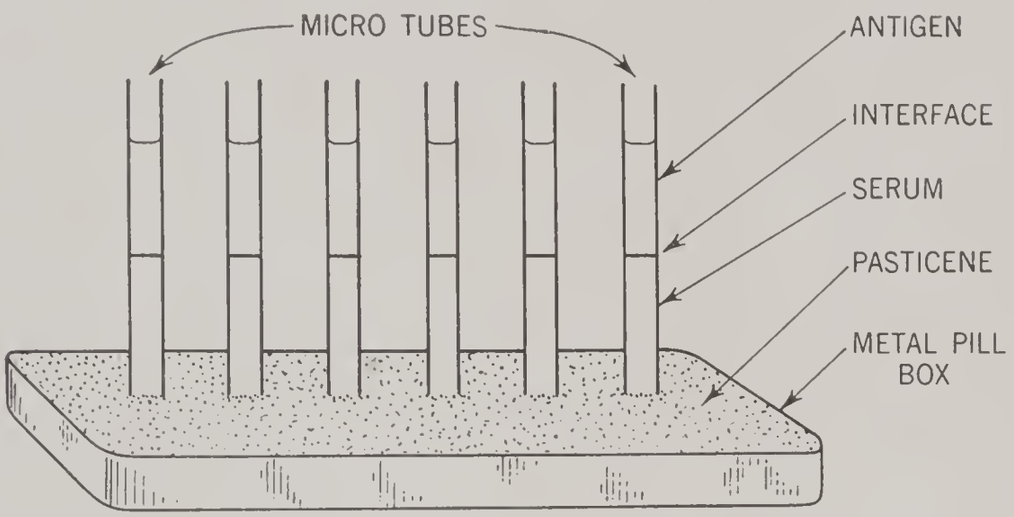

Fig. 32. Set-up for the precipitin test for human blood

The antiserum is tested with successive dilutions, for example, 1:10, 1:100, 1:1000 etc., of the homologous antigen (i.e. human serum), and the last dilution noted which gives a visible reaction in 20 minutes or less at room temperature. This is the titer of the antiserum. If desired, dilutions in smaller steps, such as 1:5, 1:25, ... or 1:3, 1:9 ..., may be tested.

A serum with a titer of 1:20,000 or over is satisfactory. However, the amount and density of precipitate, and the rapidity of the reaction, are also of significance.

3.41316 Test of Specificity of Antiserum. Before use in practice, each antiserum must be tested for specificity. If it is to be used in America or Europe in the usual type of medico-legal case, it will probably be sufficient to make sure that it does not react with any of a series of dilutions of the blood of common domestic animals, horse, dog, sheep, pig, cat, chicken, goose, etc. If it is wished to differentiate between human blood and the blood of anthropoid apes and monkeys, it will be necessary to test the antiserum with these bloods also. It is probable that a reaction will be

found with the bloods of the higher apes. In some cases also a reaction will be obtained with low dilutions of various mammalian bloods (mammalian reaction of Nuttall). Some antisera may react weakly with human milk.

3.41317 Production of a Completely Specific Antiserum. If the antiserum is found to react with any of the non-human samples tested, it may be rendered specific by specific absorption. That is, some of the heterologous antigen is added, the precipitate removed, and the absorbed antiserum is thus deprived of its power to react with this heterologous material.

The antiserum is treated with an amount of the heterologous blood serum with which it will react most rapidly ("optimal proportions" of Dean and Webb). This may be discovered by mixing equal amounts of antiserum, diluted about 1:5 or 1:10 to make the reaction slow enough to time, with equal amounts of successive dilutions of the heterologous antigen, and observing the rates of flocculation (= formation of visible particles of precipitate). The amount in the last tube is then multiplied by 5 or 10 (dilution of the antiserum used for the optimal proportions test) to obtain the appropriate amount for the undiluted antiserum. The volume added to the antiserum should not exceed $\frac{1}{2}$ its original volume. The mixture of antiserum and heterologous antigen is allowed to stand in the ice box all night, and the precipitate centrifuged off. Generally it will be found that the fluid remaining will no longer react with the heterologous antigen (or with other antigens closely related to it), while retaining a practically undiminished power of reacting with the homologous antigen. If a heterologous reaction is still found, treatment with about $\frac{1}{4}$ the original amount of heterologous antigen will usually remove it. The titer of the absorbed antiserum must then be determined afresh against the homologous antigen.

3.41318 Preparation of Material for Test. Before the reaction can be carried out, the unknown material must be brought into solution. If the blood stain is on hard material such as glass, smooth wood, metal, etc., it should be scraped off, powdered, and the powder extracted. A roughly equal amount of scrapings from the unknown material should be extracted in the same way for use as a control. If the stain is on cloth, paper, etc., the stain, or a portion of it if it is large, should be cut out and extracted. An equal portion of unstained material should be extracted at the same time.

The material is extracted with physiological saline (0.9% NaCl solution), over night in the ice box. It is suitable to use about 1 cc. of saline (more if the material is very absorptive) for each square centimeter of stained material, in the case of cloth, etc., or for each 4–5 mg. of dried blood powder. This should give a solution, if all the dried serum proteins

dissolve, equivalent to a 1:10 or 1:20 dilution of fresh serum. The actual content of dissolved protein should be estimated, simultaneously with the precipitin test, by shaking a portion to produce foam, and by placing small amounts of the successive dilutions over concentrated nitric acid in little precipitin tubes, and noting the last dilution which gives a visible precipitate at the junction. This last dilution of the extract will correspond to a dilution of about 1:1000 of fresh serum. The dilution which just forms stable foam is similar (34).

The extract should be filtered and if necessary centrifuged at high speed to clarify it.

3.41319 The Precipitin Test. Assuming that a specific, strong antiserum is available, and an unknown blood stain has been extracted and the extract rendered clear, the actual test may be set up.

Uhlenhuth recommends placing the antigen dilutions in the tubes (see above) first, then running the heavier serum in to the bottom with a pipette. Aside from this, and the larger amounts used by Uhlenhuth, the technic we describe here is not significantly different.

Using the smaller tubes described above, about 0.04 cc. of antiserum will be placed, using the capillary pipettes, in the bottom of each of nine tubes, avoiding bubbles. In another tube will be placed about 0.04 cc. of the normal rabbit serum.

Then, over the serum, in contact with it so as to form a sharp junction without bubbles, will be placed, in 5 of the antiserum-containing tubes, about 0.04 cc. of a 1:100,000, a 1:10,000, a 1:1000, a 1:100, and a 1:10, respectively, dilution of the unknown stain. For this the same pipette may be used, beginning with the most dilute solution and progressing to the most concentrated, without rinsing. When the 1:100 dilution is reached, some should also be placed over the normal rabbit serum. Then with another pipette, 0.04 cc. of saline is placed in another antiserum-containing tube, and an extract of the unstained substrate in another, in that order. With another pipette, a 1:1000 dilution of some heterologous blood serum* is added to an antiserum-containing tube, and with another pipette a 1:1000 dilution of known human serum is placed in another tube also containing antiserum. The set-up will have the appearance shown in table 43.

Purpose of the Controls. In order to be able to make positively the diagnosis of human origin of unknown material, the rather elaborate controls outlined are needed:

a. *Normal rabbit serum*, (tube 6). This is used to test if the extract of

* If there is any clue to the possible origin of the stain, assuming that it is possible that it is not human, blood of the suspected species should be used here.

the unknown material will give a (non-specific) precipitate with sera not containing anti-human precipitins. Extracts of some materials have a non-specific precipitating power when mixed with sera. The reaction in this tube must be negative.

b. *Saline*, (tube 7). This tests if the antiserum tends in general to cloud when in contact with other, non-specific fluids. This tube also serves as a standard with which to compare the appearance of the other reactions. The reaction here must be negative.

c. *Substrate*, (tube 8). This tests if the substrate has, independently of the stain, any specific or non-specific precipitating power. The result here must be negative.

TABLE 43

SCHEME FOR THE COMPLETE PRECIPITIN TEST, WITH CONTROLS

	Tube number									
	Tests					Controls				
	1	2	3	4	5	6	7	8	9	10
Upper layer	Ext., 1:100,000	Ext., 1:10,000	Ext., 1:1000	Ext., 1:100	Ext., 1:10	Ext., 1:100	NaCl	Substrate ext.	Heterologous blood, 1:1000	Known human blood 1:1000
Lower layer	Antiserum	Antiserum	Antiserum	Antiserum	Antiserum	Normal rabbit serum	Antiserum	Antiserum	Antiserum	Antiserum
Expected result						−	−	−	−	+

d. *Heterologous blood*, (tube 9). This controls the specificity of the antiserum, and shows that it does not react with mammalian bloods in general,* at least not in high dilution. The reaction here must be negative.

e. *Known human blood*, (tube 10). This controls the activity of the antiserum. It is necessary to show that the serum reacts well. This tube also serves as a standard reaction with which to compare any reaction found with the unknown.

These controls are of great importance; no one of them should be omitted.

Characteristics of a Positive Reaction. If the above technic is used, a positive reaction is seen in the form of a thin white ring, or rather plane, at or very near the junction of antiserum and antigen, which appears within a few minutes after the antigen has been added, and grows heavier. In a very strong reaction particles of precipitate may begin to fall down through the antiserum. The ring of precipitate should remain hori-

* In case the results are to be presented in court, the protocols may be more impressive if not one, but a considerable number of heterologous bloods are tested.

zontal if the tube is tipped, showing it is not dirt or contamination attached to the tube. Any reaction appearing after 30 minutes have elapsed should probably be disregarded.

The reactions should be read in a good light, with a dark background behind the tubes. A well lighted window which has a cross bar which can be used for the background, is very satisfactory.

Evaluation of Results. If the controls are satisfactory (tubes 6–9 negative, tube 10 positive), positive reactions appearing in tubes 1, 2, 3 or 4 indicate that the blood stain was of human (or anthropoid) origin. A reaction in tube 5 alone is of less significance,* unless the nitric acid test shows the original extract to have been unusually dilute. A reaction in tube 4 alone is probably significant but shows the concentration of serum proteins to be very low in the extract. In a clear cut case positive reactions will be obtained in tubes 5, 4, 3, and possibly 2 and 1.

Limits of Applicability of Test. A positive reaction, as described, with satisfactory controls, is diagnostic for human (or ape) blood. Human blood can even be differentiated from anthropoid blood by using a good antiserum specifically absorbed.

Human blood stains 15–60 years old have been identified by the precipitin test.

In case the extract of the unstained substrate causes a clouding a confident diagnosis will be impossible. Sometimes neutralizing the extract (i.e. bringing it to pH 7) may remedy this.

A verdict in the opposite sense, i.e., that a stain is *not* human blood should only be ventured if the stain is in a good state of preservation, not too old and preferably only if a good reaction can be obtained with another antiserum to some animal blood.

Dr. A. S. Wiener informs us that an antiserum for human hemoglobin is valuable as a check on the results obtained and may in some cases give a positive reaction (showing the presence of human hemoglobin) when the antisera described above fail.

References. The literature on precipitin tests is enormous. The reader wishing more detailed information will probably find sufficient in the following: (34, 12, 26, 9).

3.4132 Group Determination. If a stain is found to have been made by human blood, determination of its group may be undertaken. This problem is divided into two parts, study of the specific serum characteristics (agglutinins) and study of the absorbing power of the stain (agglutinogens).

* Particularly if the antiserum used is known to precipitate low dilutions of certain heterologous bloods.

3.41321 Detection of the Agglutinins. Landsteiner and Richter (19) showed that the isoagglutinins may often be detected in dried blood stains.

Cover Glass Methods. If little portions of the dried blood (crusts) can be obtained, the best method for detecting the agglutinins is the cover glass method of Lattes described above (sec. 3.1185). General considerations relating to this technic have been mentioned (see 2.711). As was said there, sensitive test erythrocytes should be used. They should be selected before the main experiment is set up, so that the often scanty unknown material may be used exclusively for significant experiments.

Pseudo-agglutination must be especially looked out for (cf. sec. 2.2611). Corpuscles of group O are indispensable for a control. Only very small (ca. 1 mg.) particles of dried blood should be put on the slide.

If little crusts and scales may be obtained directly from the substrate, as with metal, glass, stone and leather, they may be studied as described in sec. 3.1185. If the substrate is soaked with blood, or if a fine powder is obtained by scraping the surface, "artificial crusts" (Lattes) may be prepared. If a powder is obtained, it is rubbed with the smallest possible amount of distilled water into a paste, which is then allowed to dry. A crust is thus obtained which may be tested by the cover glass technic. Cloth and other similar substances into which the blood has penetrated are extracted with distilled water. The liquid may be forced out by squeezing, for example, between two slides. The fluid thus obtained is allowed to dry on glass in the desiccator. Then it is scraped off as a powder which is then worked over as described above.

Test Tube Reaction. If larger amounts of blood are available, extracts may be obtained which can be examined in the test tube. A small amount of the dried blood is weighed and extracted in the cold with four times the amount of distilled water. If the blood is in a hard crust, it is first pulverized. After a few hours, the material is centrifuged, and the supernatant fluid examined for its agglutinin content. Some authors (cf. (30)) recommend extraction in the refrigerator all night. The test is carried out in small test tubes as described above.

As mentioned in sec. 2.711, it is desirable after allowing time (30 to 60 minutes) for the reaction to be completed, to centrifuge and replace the supernatant by physiological saline. Then when the sediment is shaken up, the reaction is more easily read.

If there is a possibility that the blood could have deteriorated at all, only a *positive* result, that is, demonstration of the presence of an agglutinin, is of value. If the agglutinin anti-A is found, then we are dealing with blood of group O or group B. If anti-B is found, the group is O or A. The presence of both indicates group O.

If a given agglutinin is not found, it may be because the blood did not

contain it originally, or the agglutinins of the sample may have deteriorated since the stain was made. It is possible for one agglutinin to have disappeared, out of two originally present.

Various authors differ in the reliability they attribute to the test for agglutinins in blood stains. It is on the whole less likely to succeed than the test for absorptive power, about to be described.

3.41322 Detection of Agglutinogens. The presence of the agglutinable substances may be demonstrated by the absorption method. The material to be investigated, in a finely divided state, finely powdered if possible, is mixed with anti-A and anti-B agglutinins. After a suitable time the material is centrifuged out, and the supernatant fluid is tested for its content of agglutinin. Removal of an agglutinin indicates the presence of the corresponding agglutinogen. The principle is illustrated in table 43a. It depends upon the fact that if a material can remove specifically the agglutinin anti-A, for example, from a serum, we may infer the presence of the agglutinogen A in the material (see figures 18 and 33).

TABLE 43a

METHOD OF DETERMINING THE BLOOD GROUP OF BLOOD STAINS, ETC., BY THE ABSORPTION TECHNIC

Material mixed with serum containing agglutinin	Cells	Results of testing supernatant stain number			
		1	2	3	4
Anti-A	A	+	−	+	−
Anti-B	B	+	+	−	−
Inferred group of stain		O	A	B	AB

Careful controls are necessary in such work, and for greatest accuracy the tests should be done quantitatively. Greater certainty is secured if both normal human and group-specific immune sera are used.

The general technic of the absorption method has been discussed above (sec. 2.72), and the reader may review that section before proceeding. If plenty of material is available, the supernatant sera after treatment with the stained material can be titrated for its strength of agglutinins (cf. sec. 2.2211), making the dilutions in test tubes in the usual way, and the result compared with the original titer. References, and descriptions of the technic as applied to blood stains, will be found in (15, 16, 32, 33, 36, 3, 18, 7).

Since, however, the amount of material available is so often limited, we may content ourselves with describing here a technic appropriate for small samples. By using sera diluted to known strength, and counting as significant only a definite degree of removal of agglutinin, the operation is

made equivalent to a subsequent titration of the agglutinin in the supernatant, without the necessity of having enough material available for actually carrying out such a titration.

Reagents. The importance of using the proper reagents, and of knowing the peculiarities of their behavior, has been stressed in the chapter on general technic (sec. 2.7221). The reader is advised to refer back to this before proceeding. We may again mention the desirability of using absorbed immune anti-A and anti-B sera, as well as human sera of group B and group A. In addition to serving as a valuable check, as mentioned above, they make detection of A_2 stains easier, particularly if A_2 cells are used in testing, and they are less likely to be interfered with by any water soluble group substance present on the material as a contamination (37).

The strength of the sera must be estimated by titration, by making progressively doubled dilutions, which are tested under the same conditions as those under which the sera will be used. The tests may be carried out by the hanging drop method, on cover slips sealed on little chambers on slides with vaseline, or in "well slides". One loopful of serum is mixed with one of A_1 erythrocyte suspension (ca. 1 per cent), and another mixed with B erythrocytes. (Greater delicacy can be attained by using a lower proportion of cells to serum (sec. 3.6533).) The erythrocytes should be from persons whose cells have been shown to react properly. After 5 to 15 minutes the reactions are read under the low power of the microscope. The reactions should be recorded according to a consistent system.

The symbols used in table 44 are as follows: the symbol (−) signifies a completely negative field; all the cells are dispersed, giving a smooth, even appearance; as the preparation stands, the cells tend to settle down together into the center of the drop, still remaining completely separate. (±m) signifies a field in which at first there is some clumping, in large loose masses, but on standing the cells begin to fall apart, and after a few minutes a mottled field, without clumps, but with somewhat uneven distribution of the cells, is formed. The cells do not sink to the bottom of the drop readily. (±) Here there are a few clumps, not very definite, in a field otherwise like the preceding. It can be adjudged as positive or negative only in comparison with known positives, keeping in mind the strength of the serum and the behavior of the controls. (+w) This field shows small but well defined clumps, but not all the cells are involved. This is positive, but with strong sera, it may indicate some removal of agglutinin. (++) This is a clear and undoubted positive, showing large closely knit clumps in which the cells partly lose their form; it involves all or nearly all of the cells in the field, so that many spaces devoid of cells are seen.

After titration, the B serum, and the A serum if necessary (the titer of many A sera is not high enough to allow their use even undiluted) are

diluted so that they agglutinate the appropriate cells (±) when further diluted 1:8, and are negative when diluted 1:16. In other words, in the set of tubes for determining the limiting titer, begin with the first negative tube and count back four tubes. The dilution in the tube just preceding this (i.e. next strongest) is the one to use for this work. The absorbed immune anti-A and anti-B sera are diluted and mixed so that the mixture when further diluted 1:8 agglutinates both types of cells (±), but when diluted 1:16, fails to agglutinate either. In the titrations of these sera, count back three tubes and mix the dilutions found, in the case of each serum, in the immediately preceding tube. The mixing of course provides a further dilution of 1:2 immediately.

Group O serum is not used ordinarily, on account of the commonly unequal strength of the agglutinins, and the possibility that, in some O sera, the removal of one agglutinin may involve some removal of the other. Human A and B sera are not mixed on account of the possibility of some neutralization of the agglutinins by A and B substances normally occurring in the serum (see sec. 2.3242). (According to Wiener (35), with dilute sera this factor is unimportant.)

The final diluted mixtures are again titrated to check their strength, and adjusted if necessary. They may be preserved from bacterial decomposition by "Merthiolate" or acriflavine, but the diluted agglutinins do not appear to keep much better when so preserved than when kept sterile without preservative. They retain their strength in some cases for several weeks, in others for only a few days. They should be tested, or new mixtures made up, before any stain is tested.

These reagents must be tested with a collection of known stains, as not all sera behave alike, and the agglutinins of some sera are much more readily removed by the appropriate antigen than they are from other sera of equal strength as judged by titer alone. It is extremely important to find sera which react in the proper way, and to become thoroughly acquainted with the behavior of the particular sera being used.

Procedure. The actual determinations are carried out as follows. An amount of stained material is taken which has on it about 4.5 mg. of dried blood (equivalent to about $\frac{1}{3}$ drop of fresh blood). Using the material from a man's shirt, for example, this is about 1.1 sq. cm. of stained material. Such a sample, and two similar amounts, are placed in three small test tubes (ca. 2 cc. capacity). In one tube is placed 0.1 cc. of diluted A serum, in the second the diluted B serum, and in the third the diluted mixture of anti-A and anti-B sera. The mixtures are thoroughly mixed and prodded, so that the material becomes wet, with a wire or thin glass rod, left in the ice box for 24 hours, remixed and tested, mixed and prodded again, replaced in the ice box for a further 24 hours, then again tested.

The amount of serum and stained material should be such that there is just sufficient excess of serum to enable a few loopfuls to be taken off for testing. If it proves difficult to obtain any liquid from a test using the above amounts, a little may usually be drawn up through a micro-pipette pressed against the material. The supernatants are tested with A_1 and B cells as described above.

Along with the unknown stain there must be tested simultaneously, and with the same sera, the following controls, preferably made on similar material: unstained material, stains of group O, A_1, A_2, B, A_1B, A_2B. These controls are *absolutely necessary*; no one of them can be dispensed with. They should if possible be of an age similar (within a few months) to that of the unknown stain.

One essential control, which strangely enough is not always mentioned by writers on this subject, is to test the unstained portion of the material on which the stain is found (cloth, paper, wood, etc.) for group-specific absorptive power, before undertaking to test the stained portion. Several authors have pointed out (5, 13, 22) that in a surprisingly large number of cases this unstained material will contain one or both of the blood-group receptors, presumably from sweat, urine, animal material, etc., and consequently a positive test for this receptor in the unstained portion would mean nothing. If only one receptor is found in the unstained material, tests may be carried out to detect the possible presence of the other one, though it will readily be appreciated that in such a case we cannot expect to establish the full and exact group of the stain. The unstained material should be cut off from as near the stain as possible.

Conclusions. The results of the tests of the supernatants from the unknown stain must be interpreted in the light of the results given by the control stains. If the anti-A agglutinin is completely removed, *from both the human and immune sera*, the stain may be classified as group A, providing the control A behaves similarly. Stains of group A_2 may not always effect quite complete removal, but will markedly reduce the strength of the reaction. If only partial removal is achieved, the stain and controls O, A_1 and A_2 should be retested with an anti-A serum which reacts (±) at 1:4, and the behavior of the four stains compared to determine the correct grouping of the unknown. If the anti-B is removed, also from both kinds of serum, the stain is a B. If both agglutinins are removed, the stain is an AB. In the case of A_2B stains, some residual anti-A is often left, and these stains should be retested with weaker serum.

If no agglutinin is removed (i.e., all sera agglutinate practically as strongly as at first), the stain may be provisionally classified as group O, but it must be remembered that there is always the possibility that one or more of the agglutinogens was originally present, but has disappeared, or

lost its reactive power; in very fresh stains a test for agglutinins may decide. The studies of Therkelsen (32, 33) show that this does not readily occur under ordinary conditions (see below), but the possibility must be kept in mind. Only in case a number of stains of equal age, exposed to similar conditions, are typed and some are found still to be reactive, or in case receptor M or N can be demonstrated in an otherwise negative stain,

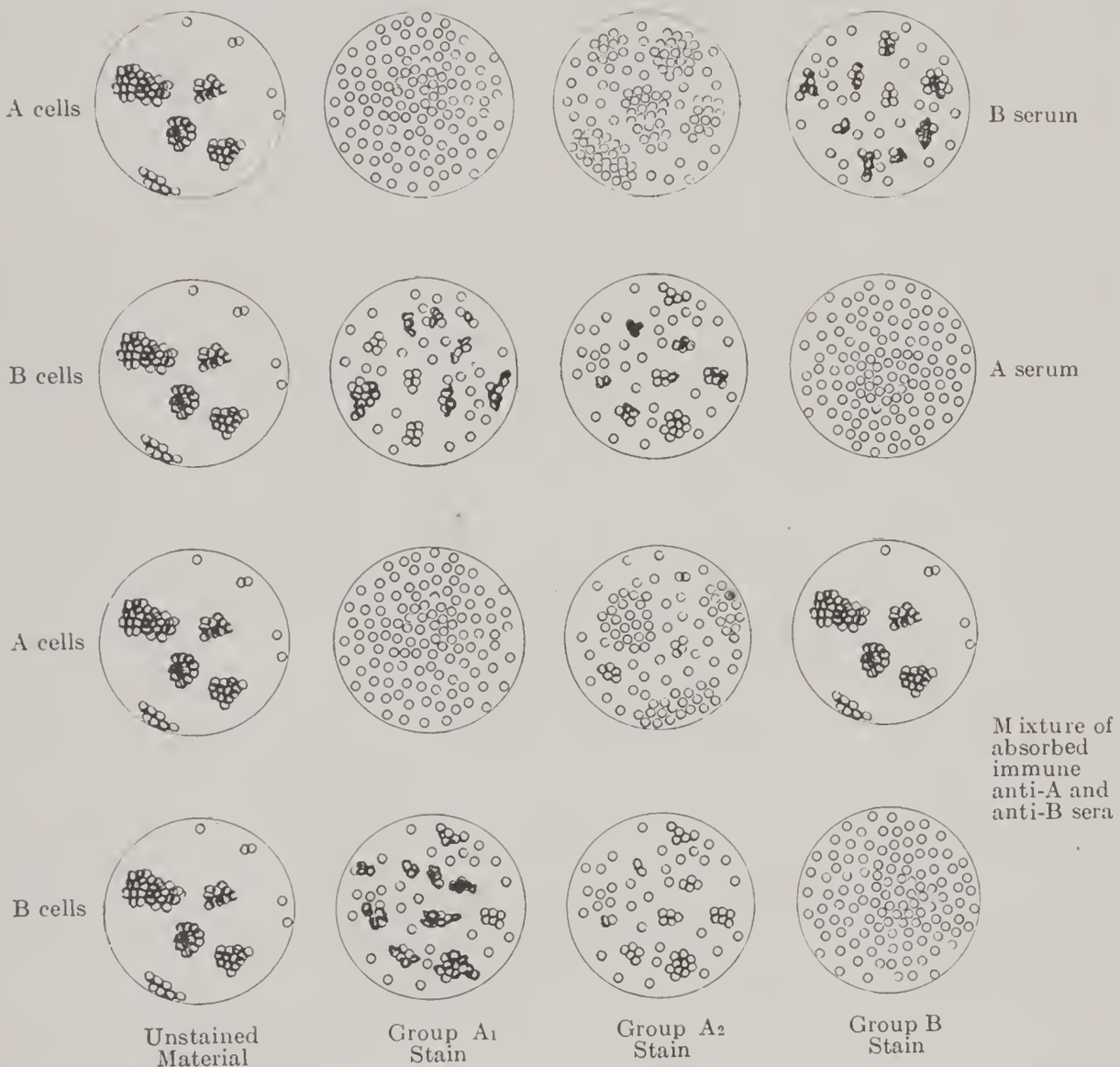

Fig. 33. Absorption of agglutinins, tested on slides (grouping of dried blood stains)

or in case one or more of the agglutinins can be demonstrated, can the operator with any confidence pronounce the stain to be of group O.

A diagrammatic representation of the group determination of stains is shown in figure 33.

The possibility of missing the A_2 factor must be remembered, though this will rarely happen with the above technic. If the existence of the very rare factor A_3 is confirmed, then in most cases it will have to be stated in reporting a stain as probably group O, that it could also perhaps, but not likely, be of group A_3.

If there is sufficient material all the tests should be repeated. Additional tests, using A_2 as well as A_1 cells for testing, are quite valuable, especially in detecting the A_2's (37). If there is sufficient stained material, a set-up may be made with larger amounts of stain and serum, (including unstained material as a control), and the degree of absorption determined by titration. The technic is described in sec. 3.65221.

It is clear that these are methods for the detection of the various agglutinogens, and not direct tests for the blood groups as entities. Consequently the report must take the form that such and such an agglutinogen has been demonstrated in the stain, rather than that the stain is group so and so. The confidence with which we make a statement will vary somewhat with what we assert. We shall be more confident in stating that a stain did not contain agglutinogen A_1 than we shall in stating that A_2 was absent, since the latter gives weaker reactions, and is somewhat easier to miss. We shall be loath to state that the receptor A_2 is absent from a stain containing B, for A_2 in the presence of B gives exceptionally weak reactions. Failure to allow for the existence of the subgroups of A probably accounts for some of the errors reported. We shall rarely, if ever, state that a stain apparently of group B could not be an A_2B.

We shall never make the statement that a stain belonged to group O, merely because no agglutinogen A or B was demonstrated. If we do state that it is probably O, we shall add that if it is not group O, its most probable group is A_2 or A_3. In general, we shall make only the statement justified by our data, interpreted in the light of the controls, and nothing further. The statement, based upon a partly inconclusive test, that a stain contains the receptor B, and possibly A_2, may be of some value in certain circumstances, but to hazard the guess that the stain is group B or AB, may be incorrect and misleading. If the experiments are inconclusive, the experimenter will refrain from giving an opinion.

Using this technic, Boyd and Boyd (3) determined the groups of 150 stains without error. Among these were 28 unknown stains, identified only by number, sent them by Wiener. As an illustration of the sort of results obtained, see table 44. According to Wiener, all the groupings were correct, even the identification of the A_2 bloods; he stated "There is no doubt that in competent hands and when the material is suitable the determination of the group of dried blood stains is entirely practicable."

The stains reported in table 44 were only a few weeks old when examined, but it has been shown (32, 33) that the agglutinogens are fairly stable over a period of years. Holzer (16) determined the group of 100 stains, some as much as 40 years old, with results which agreed well with the known frequency of the groups in the city where the stains had originated.

Table 44*

Results of Tests on Unknown Blood Stains**

Number of stain	Immune serum		Human serum		Reported group	Actual group
	A cells	B cells	A cells	B cells		
On cloth						
1	++	++	++	++	O	O
2	++	−	++	−	B	B
3	−	++	−	++	A	A_1
4	+w	++	±m	++	A (prob. A_2)	A_2
5	++	−	++	−	B	B
6	++	−	++	−	B	B
7	−	++	−	++	A	A_1
8	−	++	−	++	A	A_1
9	−	++	−	++	A	A_1
10	−	++	−	++	A	A_1
11	++	−	++	−	B	B
12	++	++	++	++	O	O
13	++	++	++	++	O	O
14	+w	++	±m	++	A (prob. A_2)	A_2
On paper						
1	−	++	−	++	A	A_1
2	−	++	−	++	A	A_1
3	+	−	+	−	B	B
4	+	−	+	−	B	B
5	+	++	+	++	O	O
6	+	++	+	++	O	O
7	−	++	−	++	A	A_1
8	−	++	−	++	A	A_1
9	−	++	−	++	A	A_1
10	+	++	+	++	O	O
11	+w	++	±m	++	A (prob. A_2)	A_2
12	−	++	±	++	A (prob. A_2)	A_2
13	++	−	++	−	B	B
14	++	−	++	−	B	B
Controls						
Unstained cloth	++	++	++	++		
Known stain						
O	++	++	++	++		
A_1	−	++	−	++		
A_2	±	++	±	++		
B	++	−	++	−		
A_1B	−	−	−	−		
A_2B	±	−	±	−		

* (Modified from (3) in the light of a later communication from Wiener.) **Sent by Wiener to Boyd and Boyd.

For meaning of symbols, see text.

For *methods of preservation* of the results of tests carried out in important forensic cases, see sec. 3.37.

3.41323 M and N in Stains. Although there have been a number of attempts to devise methods for the detection of M and N in stains (32, 36), it does not seem that any technic sufficiently reliable for medico-legal application has been found in the case of the agglutinogen N. Methods for detecting M are apparently more reliable (21, 32). An absorption method has been described by Lauer (21), which makes use of a device for measuring the amount of shaking required to give agglutination with absorbed and unabsorbed sera. This apparatus might find application also to the determination of A and B in stains. Details will be found in (21). Lauer's technic has been applied by him to the detection of both M and N in stains, some in medico-legal cases.

We shall confine ourselves here to the technic of Therkelsen for the detection of the agglutinogen M (32).

Apparently not all absorbed anti-M sera are suitable for the absorption experiments. Therkelsen recommends that before undertaking a real investigation, a number be tested until a satisfactory serum is obtained. It should have a titer of 1:32 to 1:64.

Supposing the stains to be on cloth, cut off a stained piece weighing 50 mg., and an unstained piece weighing 35 mg. as a control. Use as other controls 50 mg. pieces of cloth stained with blood of type OM, OMN, and ON, respectively. These samples are placed in small test tubes, and to each is added 0.15 cc. of a 1:2 dilution of absorbed anti-M testing fluid. The samples and serum are carefully mixed, and allowed to stand $1\frac{1}{2}$ hours at room temperature. Then a small drop is removed and tested with OMN cells. It will be found that agglutination is obtained with the fluid from the unstained material and the N stain, but not from the others.

Then 0.1 cc. of a 1:2 dilution of the anti-M is added to each tube, mixed, and allowed to stand $1\frac{1}{2}$ hours. Then a small drop is tested as before. This successive addition and testing is continued, until the MN control stain shows some agglutination remaining. Then the supernatants of all the tubes are removed and their titers determined, making successively doubled dilutions in small test tubes (cf. sec. 3.65221). There may be found some non-specific absorption by the unstained material (2 stages lowering of titer), but the M stains should be found to have removed practically all of the agglutinin, and the MN stains to have removed all but a stage or two. A decision between M-containing and M-free blood is usually not difficult.

As has been mentioned, the demonstration of M in a stain which does not show absorptive power for anti-A or anti-B may serve to confirm the diagnosis of group O.

3.42 Material Other Than Blood

3.421 Organs and Tissue Fragments

Cells other than erythrocytes are not, with the doubtful exception of leucocytes, directly agglutinated by group specific sera. Nevertheless, practically every cell and tissue fluid of the body contains group substances in conformity with the individual's blood group (see table 45). Two methods are available for the demonstration of the group properties in

TABLE 45

GROUP SPECIFIC SUBSTANCES (28, 27a, 25) A AND B FOUND IN

Fluids (of "secretors")			Organs		
Plentifully	Sparingly	Not found	Plentifully	Sparingly	Not found
Spermatic fluid	Serum	Cerebro-spinal fluid	Erythrocytes	Testicle	Lens
Gastric juice	Plasma		Leucocytes	Brain	Placenta**
Duodenal juice	Urine		Spermatozoa	Feces	Hair
Bile			Muscle	Skin	Compact bone
Small intestinal contents			Stomach	Fat	Cartilage
Amniotic fluid (child's group)			Intestine	Cancellous bone	Epithelial cell of skin
Vaginal secretion			Spleen		Nails
Sweat			Pancreas		
Saliva			Kidney		
Tears			Adrenal		
			Aorta		
			Heart		
			Lung		
			Tumors		
			Placenta*		
			Liver		
			Prostate		
			Gall bladder		
			Submaxillary gland		
			Oesophagus		

* Amnion contains child's agglutinogen(s); decidua, mother's agglutinogen(s).
** Chorion frondosum.

organs, (a) the absorption technic, and (b), inhibition and complement fixation tests using extracts. The M and N factors do not seem to occur in the body outside of the erythrocytes.

3.4211 Absorption Technic. Finely ground tissue can be suspended in saline and used fresh, but it is probably preferable to dry the material, in amounts of 5 to 20 g., in a desiccator over NaOH or $CaCl_2$. Then the samples are ground fine in a mortar, and weighed amounts used for the tests.

If limited amounts of material are available, or if it is wished to use only the simplest technic, the material may be tested with A and B sera and/or absorbed immune anti-A and anti-B, of such strength that the undiluted fluid agglutinates strongly; moderately diluted 1:2, weakly at 1:4, and not at all at 1:8; thus complete removal of agglutinin by the tissue will correspond to a titer reduction of 3 tubes. The immune anti-A and anti-B agglutinins may be used in the form of a mixture in which each agglutinin has the above strength. It is preferable to use the human sera separately.

0.05 g. samples of dried ground material are placed in test tubes and mixed with 0.3 cc. of the testing fluids. The supernatant is tested with the appropriate erythrocytes (A and B for the mixed immune anti-A and anti-B, B for the human A serum, and A for the human B serum), then

TABLE 46

TESTS FOR A AND B IN DRIED MUSCLE (1)

Fluid for adsorptions	Test cells	Source of muscle									
		A	A	B	B	O	O	O	O	O	O
Anti-A	A	−	−	+	+	+	+	+	o*	+	+
Anti-B	B	+	+	−	−	+	+	+	o	+	+
B serum	A	−	−	+	+	+	+	+		+	+
A serum	B	+	+	−	−	+	+	+		+	+

* When cells were added to the supernatants from this sample, they lost their form and disappeared before a reading could be made. (Cf. sec. 3.6533.)

retested after 24 hours in the icebox. Typical results are shown in table 46 (1).

If more material is available, or if it is wished to confirm results obtained by the foregoing technic, the agglutinin removal may be gauged by titration. The testing fluids should be somewhat stronger, with titers between 1:8 and 1:32. About 0.4 cc. of each fluid is placed on about 0.08 g. of dried ground material. Controls prepared from material of known blood group should be included. After mixing and 48 hours standing, the supernatants are removed and titrated as described in sec. 3.65221, using 0.1 cc. pipettes to make the dilutions.

It has been shown (2) that the group properties are demonstrable by the above technic in the organs of non-secretors as well as secretors (cf. sec. 1.43 and sec. 3.42122).

3.4212 Tests of Extracts. Schiff (28) showed that it was possible to extract the group specific substances from the tissues in two forms, one

soluble in water, the other in organic solvents such as alcohol and chloroform. Such extracts will inhibit specifically the action of group specific sera.

3.42121 Aqueous Extracts. Aqueous extracts are prepared as follows (28): 5 g. of the fresh organ thoroughly cleaned is cut into small pieces and ground with sand, 25 g. of distilled water being added. The mass is then boiled for an hour and centrifuged. The supernatant fluid is evaporated and the residue dissolved in a quantity of saline equal to half the weight of the fresh material employed.

The sera to be used are diluted to the proper strength, which is determined by Hartmann as follows (11): successively doubled dilutions of the sera are set up, 0.1 cc. remaining in each tube. To each is added a drop of a 2 per cent cell suspension of the appropriate group. The degree of agglutination is read and recorded on a numerical scale. The average values of agglutination obtained in duplicate experiments are plotted against the dilutions. The working dilution taken for the experiments is that at the beginning of the downward trend of the curve towards less complete agglutination.

A series of successively doubled dilutions of the extract is prepared, and to each is added an equal volume of the working dilution of the serum. It is convenient to use 0.1 cc. of diluted extract and 0.1 cc. of serum dilution, making the final volume in each tube 0.2 cc. The tubes are allowed to stand at room temperature for an hour; then to the tubes containing anti-A agglutinin there is added A_1 cells (1 drop of a 5 per cent suspension), and a similar amount of B cells to the tubes containing anti-B. The reactions are read 2 hours after adding the cells. The number of tubes in which agglutination is lacking constitutes the titer.

It has been found that, although organs of some non-secretors are not absolutely devoid of agglutinogen (10, 11), and there is some overlap, on the whole the difference between secretors and non-secretors observed in their salivas is paralleled by the content of group-antigen extractable by this method.

3.42122 Alcoholic Extracts. These are prepared as follows (11): about 5 g. of fresh organ is washed and ground in a mortar. To this is added 50 cc. of 99 per cent alcohol, and the mixture kept for about 10 days at room temperature, then filtered. An aliquot portion of the filtrate is evaporated to dryness and suspended in an equal volume of saline.

Extracts produced in this way may be tested by the inhibition technic precisely as described previously.

The complement fixation technic can also be used to study alcoholic extracts. In this case the alcoholic filtrate is not evaporated, but mixed

with an equal volume of saline. This constitutes the "antigen"; the serum is one produced by immunization of rabbits with human A_1 blood corpuscles (see above sec. 2.32).

After incubation of several successively doubled dilutions of the serum (0.1 cc.) with 0.1 cc. of "antigen" and 0.1 cc. of complement for 1 hour at 37°, 0.2 cc. of sensitized sheep cells are added, and the degree of hemoly-

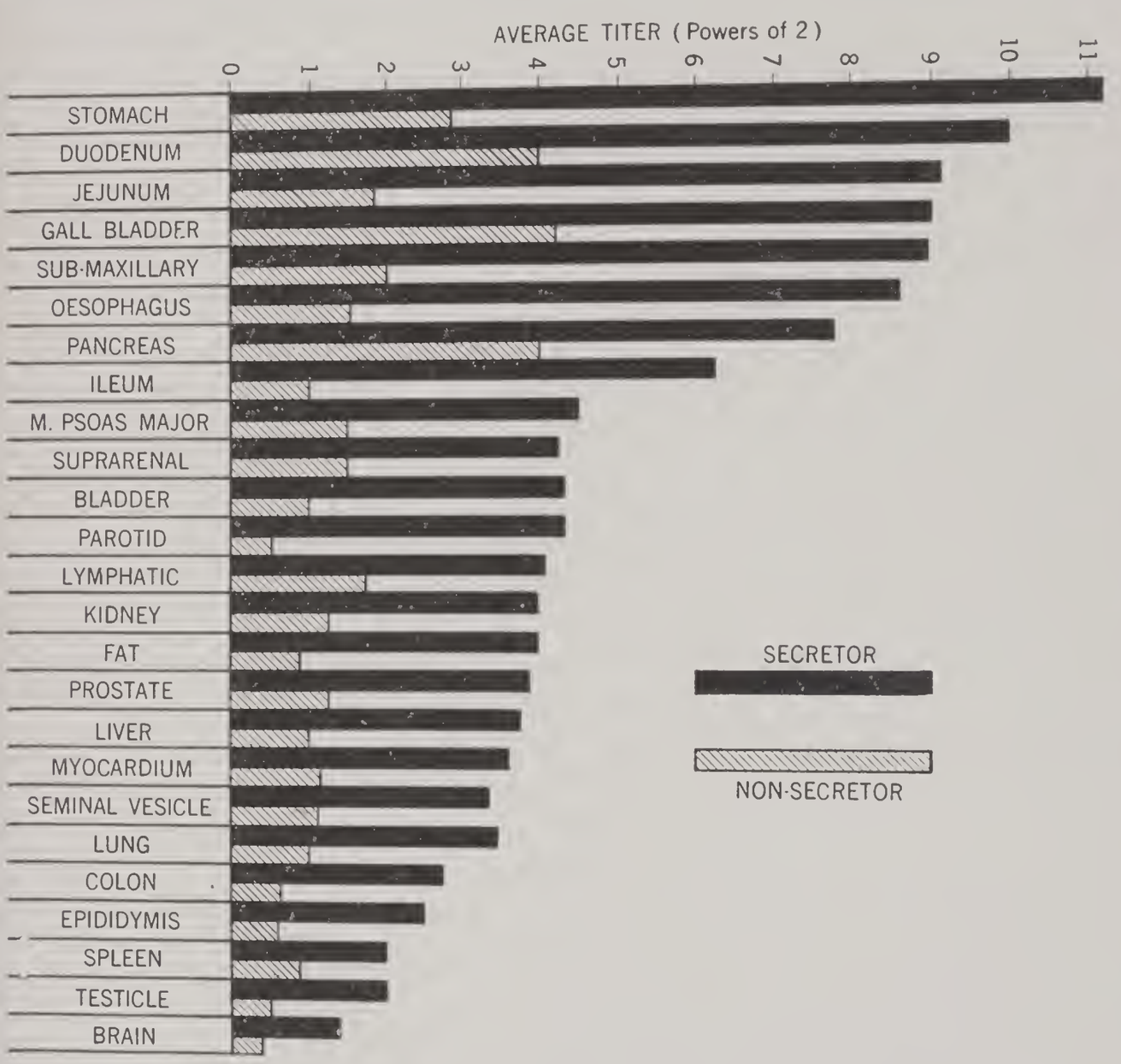

Fig. 34. Average group antigen concentration in 25 organs of the human body (Hartmann)

sis read after the usual incubation at 37°. In this case the titer is the highest dilution of the anti-A serum which shows fixation of complement (no hemolysis of the sensitized sheep cells).

In contradistinction to what was observed with aqueous extracts, it has been found that alcoholic extracts of organs do not show any difference between secretors and non-secretors. Furthermore, preliminary aqueous extraction of organ material does not diminish the amount of alcohol-

soluble group antigen which can be extracted from it (10, 11). It thus seems that the two sorts of group substances, alcohol soluble and water soluble, belong to two entirely different systems (11). In the red corpuscles only the first is found, in the organs of non-secretors only the first, and in the organs of secretors, both. In most of the secretions of the digestive system (saliva, gastric juice and bile), the water soluble antigens only are found, but in considerable amounts. In non-secretors only insignificant amounts are found.

The alcohol soluble antigens occur in about the same concentration in all organs, except the brain, the colon, and probably the fat.

TABLE 47

RELATIVE AMOUNTS OF GROUP SUBSTANCE IN EXCRETIONS AND SECRETIONS OF INDIVIDUALS OF THE "SECRETING TYPE," COMPARED WITH ERYTHROCYTES (11, 27a)

Material	Dilutions which inhibit isohemagglutination
Gastric juice	1:200–200,000
Saliva	1:100–2000
Sperm	1:100–1000
Bile	1: 30–65,000
Amniotic fluid	1: 64–256
Erythrocytes*	1: 8–32
Tears	1: 2–8
Urine	1: 2–4
Cerebrospinal fluid	0

* Tested directly in 50% concentration.

3.422 Secretions and Tissue Fluids

Body fluids can be tested by the same methods used for organs. The samples are prepared by boiling about 20 minutes, then centrifuging. The supernatant liquid is used for inhibition tests. Hartmann (11) did not find any evidence of alcohol soluble antigens in saliva, gastric juice, or bile. Sperm contains alcohol soluble antigens, in somewhat lower concentration than the organs. These fluids all contain (in secretors) considerable amounts of water soluble antigen. The relative antigen concentration in various body fluids is shown in table 47.

3.4221 Urine. Urine from secretors contains a small amount of group substance, enough to make possible a diagnosis of the blood group of such persons (11).

3.4222 Serum. Serum contains the group antigen demonstrable by the inhibition technic (sec. 2.75) or the precipitin reaction (sec. 2.3242),

and there seems to be a difference in the antigen content in secretors and non-secretors (11, 29).

3.4223 Pericardial Fluid. The pericardial fluid contains group substance, as well as some agglutinin (sec. 3.4231).

3.4224 Stains. In the case of dried stains, or material resulting from any of the body fluids, the inhibition (absorption) test as applied to dried organ preparations (sec. 3.4211) may be attempted. In this way certain workers have reported being able to determine the smoker's group (if he is a secretor) from a cigarette butt. It is not impossible that the saliva left on the flap of a gummed envelope may be sufficient (6, 20).

3.4225 Semen. The investigation of seminal stains is of special importance because of its connection with cases of actual or attempted rape. The tests may be carried out as described above for blood stains. Therkelsen (32) recommends that a diagnosis of group O, based on the absence of absorptive power for anti-A and anti-B agglutinins, should not be made unless the Florence test, carried out on another sample of the stain, is positive. He points out that in any case where it is attempted to compare the group of a stain other than a blood stain with the group of a certain person or persons, it is necessary to produce from the latter not only blood, but also saliva, to determine whether the individual is a secretor or not. The saliva should either be investigated promptly or heated to 100° for 10 minutes within an hour after the saliva is obtained.

3.4226 Feces. The problem of identification of feces occurs rather frequently in forensic medicine. An "individual diagnosis" has been suggested on the basis of serological differences in the individual B. coli strains present, but this method has not found practical application. Microscopic examination may occasionally be helpful (in one case an unusual type of stained wool was found, and the same material later found in the stools of a suspect. It was traced to material he had put in the cavity of a tooth). The determination of the blood group from feces has the same possibilities and limitations for forensic medicine as it has in the case of blood stains.

Feces stains or blood stains soiled with feces or other excretions may come up for examination. Moharrem (25) has described a reliable procedure, an improvement of one proposed by Hodyo (14). The group substances A and B are largely destroyed in descending the digestive tract (blood group enzyme, see table 48). This is not due to the ordinary digestive enzymes, such as pepsin or trypsin. Sterile filtrates of stool suspensions (human and animal) have this action, lose it after being heated. The O property is destroyed in the same way. Saliva also has some enzyme action, but the effect is bound to the cellular debris if the autolysis is prevented by cooling immediately after collecting the saliva.

The decomposition of the group specific substances in the feces is however not complete. The content in group substances of the feces is about the same as that of the red cells, which means it is much lower than that of the contents of the upper parts of the intestinal tract. Both alcohol soluble and water soluble antigens seem to be present in the feces, even of non-secretors.

TABLE 48

DECREASE IN AMOUNT OF A SUBSTANCE AND INCREASE IN BLOOD-GROUP-PROPERTY-DESTROYING-FACTOR IN DESCENDING THE INTESTINAL TRACT

Inhibiting titer vs. sheep hemolysis, and inhibiting titer of peptone after treatment with contents of:

Property tested	Part of intestinal tract							
	Jejunum	Middle ileum	Lower ileum	Cecum	Sigmoid	Ascending colon	Descending colon	Control (peptone)
A substance	68125	15625	625			25	<5	
"Blood group enzyme"	32		4	<2	<2			32

TABLE 49

DEMONSTRATION OF GROUP PROPERTIES OF STOOLS BY INHIBITION TESTS

Amount of stool preparation	Anti-A serum and A cells				Anti-B serum and B cells			
	a	b	c	d	a	b	c	d
0.02	−	−	−	+++	+++	−	−	−
0.01	−	−	(+)	+++	+++	−	−	−
0.005	−	−	+	+++	+++	−	−	−
0.0025	−	−	++	+++	+++	+	−	−
0.00125	−	+	+++	+++	+++	++	++	(+)
0.000625	+++	+++	+++	+++	+++	+++	+++	++
0.0003125	+++	+++	+++	+++	+++	+++	+++	+++
Saline (control)	+++	+++	+++	+++	+++	+++	+++	+++

The blood groups of the above individuals were: a, A_1; b, A_1B; c, A_2B; d, B.

Technic. After extraction of the dried material with alcohol and chloroform, watery extracts are prepared and tested (as above) by means of the inhibition test against group specific isoagglutinins. A careful titration of the sera is important. In the case of A substance, the findings may be checked by testing alcoholic extracts for inhibition with A specific sheep hemolysin.

Preparation of extracts. 20 g. of feces are emulsified in double the amount of distilled water and filtered through gauze. The filtrate is evapo-

rated at about 50 to 60°C. The residue is pulverized and completely dried in the desiccator over $CaCl_2$. The final weight is about 1 gram. This material is extracted under a reflux condenser with 30 cc. of absolute alcohol for 4 hours at least. After centrifuging, the supernatant ("alcoholic extract") is separated and the sediment similarly extracted with 30 cc. of chloroform. This extract is filtered through paper and the residue extracted with 5 cc. of physiological saline in a steam sterilizer (100°) for one-half hour. This extract is separated from the residue by centrifuging. All residues are thoroughly dried in the desiccator before each new extraction. The alcoholic extract to be used for the hemolysin inhibition is evaporated on the water bath. The yellowish-brown material is taken up in 5 cc. of absolute alcohol. Insoluble particles are eliminated by centrifuging. Dilutions are made by slowly adding physiological saline.

In table 49 is shown an example of an inhibition test performed with the water soluble extracts from feces (after Moharrem).

3.423 Determination of the Blood Groups of Corpses

It is sometimes desired in forensic cases to determine the blood groups of persons who have been dead for shorter or longer periods of time. Up to 24 hours after death there is no particular difficulty; a sample of blood can be withdrawn and tested as already described for the living.

But when decomposition is more or less advanced, the problem is less simple. A number of methods may be applied. The possibilities are shown in the following scheme:

Blood Grouping Corpses by Investigation of:

3.4231 The classical four blood groups.
- a. The blood (cells and serum).
- b. Pericardial fluid (isoagglutinins).
- c. Organs (stomach, heart, muscles, kidneys, skin); (group antigens only).
  - *1.* by absorption directly (sec. 3.421).
  - *2.* by tests on extracts (sec. 3.4212).
- d. Bones (see below, sec. 3.6523).

3.4232 A_1 and A_2 (from cells).

3.4233 Secretion type (gastric juice, urine).

3.4234 M and N type (erythrocytes).

More detailed explanations follow.

3.4231 A, B Blood Groups. The agglutinins and agglutinogens were investigated by Palmieri (27), in some cases repeatedly, in 77 corpses, kept at temperatures ranging from 8 to 20°. The agglutinins in the serum and pericardial fluid began to decrease two or three days after death, and decreased gradually until in 15 to 20 days they had disappeared from cadavers kept in the air at temperatures between 15 and 20°. The anti-A agglutinin, usually stronger originally, persisted longer than anti-B. Pal-

mieri thinks the agglutinins probably persist longer in buried corpses. The agglutinability of the corpuscles decreased more rapidly at first, but the corpuscles were in some cases still specifically agglutinable after 12 to 15 days. The serum in all cases became pan-agglutinating (agglutinated corpuscles of all groups) in 10 to 15 days.

Other references may be found in Steffan (31).

If the cells have begun to hemolyze, a usable suspension may often be obtained by careful washing with physiological saline; they should be tested for pan-agglutinability (sec. 2.2613) before being used. When the condition of the blood is poor, an extract of the clot may be made and tested for the presence of A and B by the inhibition technic (cf. sec. 2.75 and sec. 3.4212).

In some cases, as following death by fire, the pericardial fluid may be the most readily obtained material to examine for agglutinins. After the chest has been opened, the pericardial fluid is withdrawn with a syringe. If it is not clear, it is centrifuged. It is tested like serum. Agglutinins may often be found thus, even in young children (15).

The agglutinogens persist in organs and muscles of cadavers much longer than does the agglutinability of the erythrocytes, and may be demonstrated by absorption and inhibition tests. It has even proved possible to demonstrate by this means group-specific properties in mummified tissue, some of it over 5,000 years old (sec. 3.6522). The technic is entirely similar (2, 23) to that already described above (sec. 3.4211).

The group specific properties also persist in the bones (7, 8, 4), and may be demonstrated under appropriate conditions, by absorption (sec. 3.6523). The technic is not easy, however, and skill and patience are required.

3.4232 Subgroups. It will not be expected that the subgroups of A, or the secretion types, can be established in very old cadavers. The only technics available do not differ essentially from those already described above.

3.4233 The Secretion Type. The secretion type can in some cases be determined by investigation of the saliva, or preferably the gastric secretions (11).

3.4234 The M,N Type. The M,N factors do not appear in normal tissue,* and consequently, when they can no longer be demonstrated by direct agglutination of the erythrocytes (presumably after about 10 days), it will not be possible to demonstrate them by absorption tests on the organs and tissues, as was the case with A and B.

*Positive findings are claimed by Kosjakov and Tribulev: *J. Immunol.* **37**: 283, 1939.

3.4235 Tests of Relatives. When the body is not available, or decomposition far advanced, as well as in certain other instances, it may be difficult or impossible to establish the blood group by direct tests. In some cases examination of relatives (parents, grandparents and children) may make a decision possible. If a sample of organ tissue has been taken at autopsy and preserved in some laboratory, examination of this material may make it possible to establish the group (24).

3.4236 Limitations. As in other forensic cases, only positive findings are to be considered significant in the grouping of cadavers, and very careful controls and checks are required.

It must be remembered, finally, that an inconclusive report is better than an over-confident report later found to be erroneous.

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3.5 Related Factors in Animals. Studies on Anthropoids

3.51 Related Factors in Animals

Antigens similar to the A and B in man are found in some animals.

3.511 Horses.

For example, in the saliva of the horse there may be found antigens like A and B. A and B together, either alone, or neither, may occur, thus establishing four groups, probably hereditary, similar to those in man; but the antigens are not present also in the blood, as they are in man (11, 10). Results of observations on horses are summarized in table 50.

3.512 Forssman Antigen

A fraction of A has been found to be similar to the Forssman antigen (19). It has been claimed that a Forssman-like antigen is present in erythrocytes and organs of all groups (14), though man is generally considered to belong to the Forssman-negative group.

"Forssman antigen" is not a definite chemical entity, but a serological conception, a collective term covering substances which when injected into rabbits produce hemolysins for sheep erythrocytes. The original Forssman substance produced "heterogenetic" antibodies (9) with certain properties: no lysin for cow cells, no or almost no agglutinin for sheep red cells, antibodies absorbable by tissues containing the original F-antigen (horse and guinea pig kidney).

To these original F-antigens almost every rabbit will respond promptly. They are resistant to boiling and alcohol soluble. Landsteiner's (15) chemical examination refers to this type of material. The term "F-antigen" was used in this restricted sense by Schiff when he stated that the A-antigen is "related to but different from the F-antigen" (table 51). Several authors on the other hand use the term F-antigen for all types of substances producing sheep hemolysins, except material from sheep or closely related species. The antibodies obtained are not identical *inter se*

as the original F antibodies were supposed to be (table 52). Some data on the F-antigen are based not on immunization experiments but on ab-

TABLE 50

FREQUENCY OF OCCURRENCE OF A AND B SUBSTANCES IN HORSE SALIVA

Investigators	Number of horses with substance				
	Neither	A	B	A + B	Total
Schiff (18)	?	ca. 13	?	?	ca. 20
Landsteiner (ref. to in 7)	?	ca. 75%	?	?	?
Friedenreich and Thyssen (11)	1	44	3	4	52
Boyd (2)	5	5	2	0	12

TABLE 51

SEROLOGICAL RELATION BETWEEN A AND THE FORSSMAN ANTIGEN (ACTION OF CERTAIN RABBIT SERA)

Antigen injected	Antigen for test	
	Human group A cells	Sheep cells
Human A cells	Agglutination	Hemolysis
Sheep cells	Agglutination	Hemolysis

TABLE 52

SEROLOGICAL RELATIONSHIP BETWEEN CERTAIN STRAINS OF PARATYPHOID B BACILLI, SHEEP BLOOD, COMMERCIAL PEPTONE,* AND HUMAN BLOOD OF GROUP A (AFTER (8))

Serum absorbed with	Serum						
	Immune serum vs. paratyphoid B bacilli		Immune serum vs. sheep erythrocytes		Immune serum vs. human group A erythrocytes		Normal human serum group B (anti-A)
	Hemolyzes sheep blood	Agglutinates human blood A	Hemolyzes sheep blood	Agglutinates human blood A	Hemolyzes sheep blood	Agglutinates human blood A	Agglutinates human blood A
Paratyphoid B bacilli	−	−	+++	+++	+++	+++	+++
Sheep blood	−	−	−	−	−	++	+
Human blood group A	+++	−	++	−	−	−	−
Peptone	+++	−	+++	−	−	−	−
Saline (control)	+++	+++	+++	+++	+++	+++	+++

* The reaction of the peptone is due to the swine pepsin used in preparing it.

sorption tests. In some special cases some doubt remains whether the antibody observed was a real immune F-antibody.

3.513 "Fractions" of A and B

In other animals the erythrocytes contain antigens similar to, but distinct from, A and B. Their difference from the human A and B is shown by their failure to absorb (technic as in sec. 2.72) from certain antisera all agglutinins for human A and B erythrocytes, while removing all agglutinins to which they are susceptible.

TABLE 53

HYPOTHETICAL FRACTIONS†† OF HUMAN A STRUCTURE (23), COMPARED WITH FORSSMAN ANTIGEN

Hypothetical fraction††	Thermostable	Soluble in alcohol	Present in pepsin*	Present in sheep erythrocytes	Present in organs and serum of rabbits**§
i	+	+	+	−	+
ii	+	−	+	−	+
iii	+	+	+	+	+
iv	−	−†	−	−	−
Forssman antigen	+	+	−	+	−

* Swine and beef pepsin.
** Some rabbits.
§ Not in erythrocytes.
† Destroyed by alcohol.
†† Strictly, the expression "fraction" may not be justified except where substances can actually be separated (Landsteiner).

TABLE 54

RELATIONSHIP BETWEEN VARIOUS HYPOTHETICAL FRACTIONS OF B SUBSTANCE

Hypothetical fraction**	Present in erythrocytes of					Present in horse* saliva	Agglutinin (natural or immune) for, in		
	Man	Rabbit	Chicken	Horse	Guinea pig		Man	Rabbit	Chicken
i	+	−	−	−	−	+	+ or −	+	−
ii	+	+	−	−	−		+	−	+
iii	+	+			+		+		

* Some horses.
** See note ††, table 53.

Basing their work on a study of such related antigens, various workers have separated human A and B into several hypothetical fractions (1). To this hypothetical fractionation the same objection is possible as in the case of related bacterial antigens, although in some cases it may be justified. Typical data are shown in tables 53 and 54.

To avoid almost certain confusion between the subgroups A_1 and A_2, and B_1 and B_2, and the hypothetical serological fractions of the A and B antigens, designated by some authors as A_1, A_2, A_3 . . ., and B_1, B_2, B_3, we have here and later used small Roman numerals for the latter. Thus, A_{ii} will designate the fraction of the A structure designated as 2 by Wheeler and Stuart, and A_2 will designate the subgroup (= "A klein").

The rabbits which have fractions i, ii, and iii in their tissues produce weak anti-A sera (if any), reacting only with human cells (Aiv), (sec. 2.321). Erythrocytes of rats, pigs, cattle and sheep may contain B_{ii}, but never B_i (11). These antigens may occur also in animal saliva (table 55).

TABLE 55

"FRACTIONS" OF A AND B ANTIGENS IN ERYTHROCYTES AND SALIVA OF VARIOUS SPECIES (6, 11)

Species	Erythrocytes	Saliva
Chimpanzee[1]	A	$A = A_{iii}$
Cebus hypoleucus	B_{iii}	$B_{ii} + B_{iii}$
Cebus favellus	B_{iii}	$B_{ii} + B_{iii}$
Elephas indicus	B_{iii}	B_{iii}
Elephas africanus	B_{iii}	B_{iii}
Macropus gigantus	B_{iii}	
Dog	$B_{ii} + B_{iii}$	O
Rabbit	$B_{ii} + B_{iii}$	B_{iii}
Guinea pig	B_{iii}	B_{iii}
Horse[2]	O	B_i (+ B_{ii} ?)
Man[3] ("Mein.")	$B_i + B_{ii} + B_{iii}$	B_i
Man[3] (others)	$B_i + B_{ii} + B_{iii}$	$B_i + B_{ii} + B_{iii}$?

[1] Group A.
[2] B excretor.
[3] Group B.

Our knowledge of the A antigens of cattle has been summarized by Hartmann (13). In cattle, alcohol-soluble A is found only in organs of the digestive system, and not at all in the blood corpuscles. Water-soluble A antigen in organs and anti-A agglutinins can coexist in cattle, although capable of reacting in the test tube. Individual cows vary in the amount of A antigen found in the saliva, and this is paralleled by differences in the organs. Cow saliva reacts as well with immune anti-A from rabbits as with human serum of group B, thus differing somewhat from the A in human saliva. Hartmann believes that the genetics of the water-soluble A antigen in cattle is essentially the same as in man.

If the antigens A and B are separated into fractions, it follows that the agglutinins in various sera will also be complex. This is not implausible,

since the antibodies in a serum probably are usually somewhat complex. Typical data are shown in table 56. Any anti-B agglutinin in the sera of rats, pigs, cattle, and sheep, is said to be anti-B_i.

3.514 Function of Group Substances

It is tempting to speculate on the possible physiological function of the blood group substances, in connection with the possibility that natural selection may have influenced their frequency in certain parts of the world. In particular, the serological relationship of A to some bacterial carbohydrates suggests the (entirely unproven) idea that there might have been some selective action due to slight differential mortality to certain bacterial infections. But as yet absolutely nothing is known of any normal physiological function of the group substances, and we have no reason to think the members of any one group or type enjoy any superiority over

TABLE 56

FRACTIONS OF ANTI-B AGGLUTININ IN SERUM OF VARIOUS ANIMALS

Serum of	Agglutinins
Man (A + O) ca. 98%	$\beta_{ii} + \beta_{iii}$
Man (A + O) ca. 2%	$\beta_i + \beta_{ii} + \beta_{iii}$
Rabbit*	β_i
Chicken	β_{ii}

* And probably other mammals.

their fellows. The question is one of extreme difficulty, and it is hard to devise even a hypothetical experiment which would satisfactorily answer it.

3.52 Studies on Anthropoids

The greatest interest for students of human evolution attaches of course to the question of blood group factors in the apes and monkeys, and considerable work has been done on this subject. References will be found in (5, 4).

3.521 General

As might be expected, the higher the animal in the primate scale, the more complete the resemblance of its blood group picture to that in man. In the higher primates, substances indistinguishable from the A and B of man may be found. Dahr (5) mentions that the erythrocytes of an orang or a chimpanzee which contain A will remove from human B serum all agglutinins for human A cells, as well as all active against the primate blood. Conversely, absorption of serum of an orang or chimpanzee of group A with human B cells will remove all agglutinins, not only to human B blood, but to primate B. Further than this, absorption of the serum

of an orang of group B with human A_2 cells leaves anti-A_1 active against human A_1 cells. This experiment will not work with the serum of lower animals, such as the cow. Dahr finds that B corpuscles of the orang contain all three "fractions" of B: B_i, B_{ii}, and B_{iii}. The serum of a group A chimpanzee proved to contain, like the majority of human A sera, agglutinins β_{ii} and β_{iii}. Thus the blood groups in these primates are indistinguishable from the corresponding groups in man, as had been previously pointed out by Landsteiner and Miller (16).

In the gorilla and the monkeys (4), a substance reacting with the anti-B agglutinin, but somewhat different from B, is found. The corpuscles of the gorilla "Janet" were agglutinated only weakly with an immune anti-B serum which agglutinated human B strongly, but aqueous extracts of the submaxillary gland inhibited human A (anti-B) serum in relatively high dilutions. The serum contained α agglutinins of moderate strength. Thus the group of the gorilla was: B-like (anti-A).

From this it may be concluded that properties related to A and B are by no means restricted to mankind, but are widely distributed through the animal kingdom. There is a striking analogy with the Forssman hapten, and with the specific carbohydrates of certain bacteria. Since there is reason to think that the possible chemical variety in the carbohydrate series (to which these antigens all belong, at least in part), is less than in the protein series, this may account for the more marked interspecific reactions observed also in the present instance. The probability of two carbohydrates taken at random being serologically similar would be greater in general than that for two unrelated proteins.

3.522 Results of Anthropoid Studies

The results to date of investigations of the A, B groups in anthropoids, following the original work of Landsteiner and Miller (16), are summarized in figure 35 and table 57. This is the table published by Dahr (5), plus the data of Candela (3), and Candela, Wiener and Goss (4).

The blood grouping of monkeys has not been as extensively studied as has that of the anthropoids. Practically all American monkeys (Platyrrhina) were found to have in their corpuscles a B-like factor capable of being agglutinated by human anti-B agglutinins, but not by immune anti-B. In the corpuscles of the old-world monkeys (Cercopithecidae) this factor was not found, although the rhesus monkey (M. mulatta) possesses anti-A agglutinin in its serum (16, 4). Candela, Wiener and Goss demonstrated that the B-like factor, absent in the erythrocytes of M. mulatta, was present in the tissues and secretions. The Java macaque (M. irus) was found to have in the tissues and secretions sometimes the B-like substance, sometimes the A-like substance, and sometimes both.

The state of our present knowledge of the blood groups of the primates

is shown in figure 35, modified from Landsteiner (15). Haldane (12) has pointed out that in the genera where both A and B are found, group AB should be represented considerably more often than has been found to be the case, if mating within the genus were at random. The low AB frequencies observed suggest that there may be local geographical variations in blood group frequencies in the primate genera, analogous to those observed in the human race. The difference found by Candela, Wiener and Goss between two species of gorilla (see above) may lend support to this suggestion.

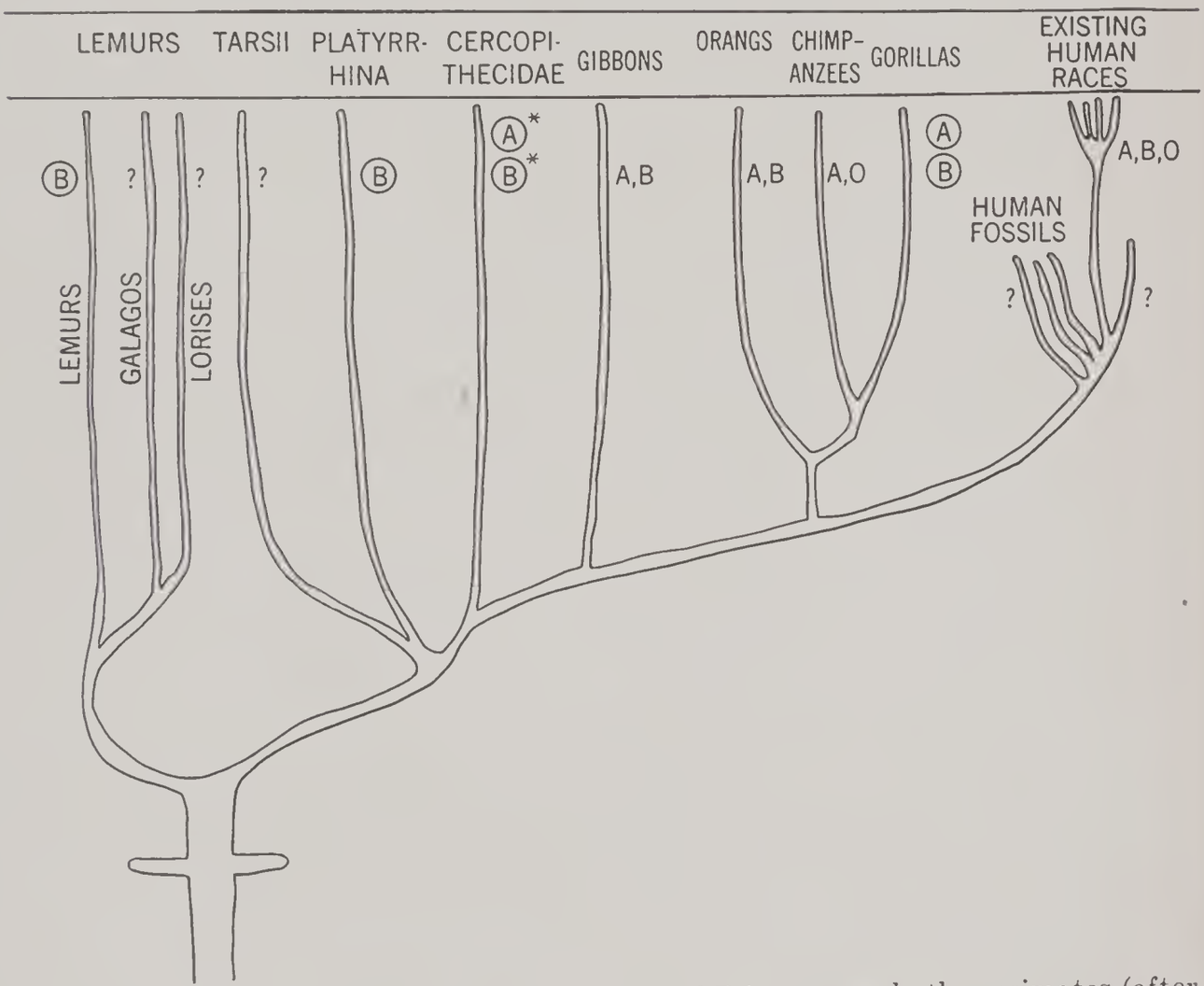

Fig. 35. Occurrence of blood factors O, A, and B in man and other primates (after Landsteiner). The symbol Ⓑ indicates the B-like antigen reacting with human anti-B agglutinins. Ⓐ* and Ⓑ* indicate A-like and B-like antigens found in the tissues and secretions.

3.523 Agglutinogen M in Primates

It has been found that the higher primates also possess blood structures capable of reacting with antisera for M, but the degree of reaction is often quite different with blood from the same animal when different anti-M sera are used. This is illustrated by table 58, based on the work of Landsteiner and Wiener (17) and Wiener (24).

To account for the behavior of man, anthropoids, and rabbits in regard to M-like antigens, hypothetical structures of the M antigen in these

TABLE 57

BLOOD GROUPS OF ANTHROPOID APES

Species	Number studied	Group				
		O	A	B	AB	?
Chimpanzee	99	12	85			2
Orang-utan	23		8	10	5	
Gibbon	15		2	7	2	4
Gorilla*	4		4?			
Gorilla (G. berengei)**	2		2			
Gorilla (G. gorilla)***	5			5		

* One of these tested by Landsteiner, who stated he was unable to assign a group; the other 3 by Weinert, whose technic (see sec. 3.5241) leaves something to be desired.

** The agglutinogen in these two bloods was "A or A-like" (4).

*** The agglutinogen in these bloods was "B or B-like" (4). Previously one of these animals was erroneously reported as an AB.

TABLE 58

REACTIONS OF MONKEY BLOOD WITH VARIOUS ANTI-M SERA (AFTER (17, 24))

Species	Anti-M serum number					
	5	1	21	35	2	82
Man (type M)	+++	+++	++±	++±	++±	++±
Man (type N)	0	0	0	0	0	0
Chimpanzee	+++	+++	+++	++	+++	±
Gibbon	++±	+±	+			
Orang-utan	++±	++	+±			
Old world monkeys (Cercopithecidae):						
Papio papio, or P. sphinx	+++	++	++±	0		0
Papio drill	+++	+++	++±	(±)	(+)	(+±)
P. porcorius	+++	+++	++±	0	tr	0
Macacus rhesus	+++	+++	++±	(+±)	±	tr
M. cynomolgous	+++	+++	+±	0	0	0
Cercocebus fulginosus	+++	+++	++±	tr	±	±
Cercopithecus sabaeus	+++	+++	0	0	0	0
New world monkeys (Platyrrhina):						
Ateles (white spider monkey)	++±	0	0	0	0	0
A. ater	±	0	0	tr		0
Lagothrix humboldti	0	0	(±)	0	0	0
Cebus sapucinus	0	0	0	0	0	0
Moss monkey	0	0	tr	±		0
Lemur catta	0	0	0	0	0	0

species have been proposed (6) which may be represented by the following formulas, where M_i, M_{ii}, etc. represent various hypothetical fractions of M: Man (type M), $M_{iii}M_{iv}M_{vi}$ . . . ; rabbit, M_{iii} or $M_{iii}M_{iv}$ or $M_{iii}M_{iv}M_v$ etc.; anthropoids, M_{iii} or $M_{iii}M_{iv}M_v$ or $M_iM_{ii}M_{iii}$ or $M_{iii}M_{iv}M_v$ etc. (Compare tables 53, 54, 55, 56.) Landsteiner and Wiener (17) are inclined to doubt the value of such a hypothesis.

It does not seem that the M factor is ever lacking in any individuals of a primate species in which it is found, indicating a difference in the genetic mechanism from that operating in man (17, 24).

3.524 Technic

3.5241 General. Investigations on the blood groups of the primates require patience and considerable familiarity with serological technic. The methods were adequately described for the first time by Landsteiner and Miller (16). Investigation of blood other than human is more complicated because the sera used are acting on *species-foreign* blood, the human serum acting on ape blood, and *vice versa*. Thus, we can no longer speak of *iso*agglutination, but are dealing with *hetero*agglutination. In view of the apparent identity of the agglutinogens and isoagglutinins in man and the chimpanzee and orang-utan, however, we may in the future restrict our use of the term heteroagglutinin to agglutinins falling outside the group scheme. It has been reported (5) that human sera regularly contain agglutinins acting on the blood of anthropoids irrespective of group, whereas the serum of the chimpanzee and the orang, at least, do not contain reciprocal "heteroagglutinins" for human blood. This means that sera of these anthropoids can be used to test human cells directly, but not *vice versa*. Weinert (20, 21, 22) tested three gorillas (see table 57) using human sera directly, and the results, according to Dahr, are to be accepted only with considerable reservations.

If a human serum of group A or group B is found to agglutinate an anthropoid's cells, we cannot without further test record the anthropoid as belonging to group B or A respectively. The finding must either be confirmed by absorption experiments, or the "purified agglutinin" solutions of Landsteiner should be used. These are solutions of anti-A or anti-B agglutinins which have been recovered from washed agglutinated human cells, and consequently are free of heteroagglutinin (cf. the *Absprengungsversuch*, sec. 2.73).

3.5242 Preparation of "Purified Agglutinins" (16). 5.0 cc. of a strong A serum (anti-B) or B serum (anti-A) is mixed with 5 drops of washed packed cells of group B or A, and allowed to stand two hours at room temperature. During this time it is well shaken several times. Then it is centrifuged, the serum poured off, and the sediment washed

three times with physiological saline. The washed agglutinated sediment is suspended in 0.6 cc. of saline and placed in a water bath at 56° for 5 minutes, and shaken several times. At this temperature the blood corpuscles give up the agglutinins bound by them. The cells are then removed by centrifuging. This must be done while the mixture is still warm, otherwise the corpuscles will again take up the agglutinin.

A large centrifuge tube is filled with water at 56° and the small tube containing the cells is placed in this; this is centrifuged rapidly for a short time, so that the majority of the cells are spun down. The supernatant fluid then contains the desired agglutinin solution and a few cells. The remaining cells may be removed by a second centrifuging without special precaution, and the clear "agglutinin solution" is obtained.

TABLE 59

ABSORPTION REACTIONS OBTAINED WITH URINE AND FECES OF THE GORILLA "GARGANTUA" (3)

Urine 0.5 cc. Test sera 1.0 cc.			Feces 0.25 cc. Test sera 1.0 cc.		
A serum	Agglutination after	B serum	A serum	Agglutination after	B serum
—	3 min.	1+	—	2 min.	1+
—	8 min.	1½+	—	5 min.	1½+
—	9 min.	2½+	1+	6 min.	2+
—	12 min.	4+	1½+	9 min.	3½+
—	30 min.	Large clumps	1½+	10 min.	4+
			2+	15 min.	Large clumps

Strength of agglutination of A and B cells by mixtures after designated times.

3.5243 Immune Sera. In studies on the anthropoids, absorbed immune anti-A and anti-B sera from rabbits may also be used, but it has been found that with the lower monkeys the reactions obtained with such sera and with the purified agglutinins do not run parallel. Therefore a difference in diagnosis may result. The immune A-specific sheep hemolysin may also be used (see sec. 2.754).

3.5244 Inhibition Technics. Another way of studying the blood groups of anthropoids is afforded by inhibition experiments, using human A and B sera, and secretions and aqueous extracts of organs. The easiest materials to obtain are urine and feces. Candela (3) has been able to examine urines from a number of gorillas. He used A and B sera of equal strength, and mixed 0.5 cc. of urine with 1.0 cc. of serum. The final dilution was thus 1:3. Typical results are shown in table 59.

This method gave correct results on a series of human urines, and

probably correct results on 5 gorillas and an orang. In the case of two gorillas, the urine, unless used in a final dilution of 1:6, gave non-specific absorption of anti-A.

Tests upon saliva and extracts of organs of primates were performed by the inhibition technic (sec. 2.75) (25, 26). In most cases it was easy thus to detect any A-like or B-like antigen present, since saliva gives much stronger inhibition than does urine. Human A and B sera were used for these experiments (sec. 2.752).

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3.6 Blood Group Investigations in Anthropology

3.61 Preliminary

3.611 General

We may assume that the purpose of anthropological classification is more serious than mere gratification of idle curiosity. If the anthro-

pologist is not content with the current popular classifications of mankind into "races" and "nations", it is because he hopes to find something more fundamental, a classification which will tell us something of the history of the human race. Thus Morant (43) says: "The main aim of physical anthropology is to unravel the course of human evolution, and it may be taken for granted today that the proper study of the natural history of man is concerned essentially with the mode and path of his descent."

If this is indeed the purpose of physical anthropology, the sort of classification we make for mankind will be one which we think will afford the most information about human evolution (9).

3.612 Requirements for a Valuable Criterion of Classification

For a scheme of classification to be of much value in reconstructing the previous history of a species, it must be based on criteria which fulfill all or most of the following conditions:

(a) The criteria must be objective; that is, the position of a given individual in the scheme must be the same, or very nearly the same, when he is classified by different observers. This rules out all, probably, of the suggested psychological and mental differences between human races, although it is possible that in the future objective methods of measuring such differences may be found.

(b) They must not be subject to too much modification by the environment; that is, they must not be so much influenced by the individual's surroundings that his innate constitution is masked. This restricts us to inherited differences, which we know to be determined by genes. Of course the effect of some genes does vary with the environment, but in a regular and predictable way.

(c) They must be determined by one, or a small number of genes. If this is not true, we shall in general find it impossible to obtain sufficient information to enable us to predict the result of crossing two different races, and shall find ourselves classifying together two or more races with significantly different makeups. It will clearly be impossible to unravel the history of large groups of genes, and their numerous permutations and combinations, over the long history of human evolution. (If genetic analysis is possible, of course, the larger the number of genes the better.) Many characteristics in present-day use by anthropologists are unquestionably the result of statistical summation of the activity of many cells and genes. Genes producing clearcut ("present or absent") effects are to be preferred.

(d) They must be non-adaptive; that is, they must not have any great selective value in evolution. If a character has selective value, it will be subject to increase or decrease in frequency in the population by the action

of selection, and the rate of this selection may be surprisingly great. Such characters can be useful only for determining recent prehistory.

(c) They must not mutate at too high a rate. If subject to mutation, a characteristic may suffer changes of frequency such as to render it useless in the study of the more remote history of the race.

3.613 Human Characteristics in the Light of These Requirements

The genetic mechanisms involved have been cleared up completely in the case of the blood groups, fairly well in the case of taste reactions. Something is known about the inheritance of hair and eye color, but our information is not complete. Other characters, such as head form and stature, are evidently determined by a considerable number of genes. It is also open to doubt if they are unaffected by the environment. We must conclude that at present only blood groups and possibly taste reactions to phenyl-thio-carbamide can be expected to meet our five stipulations (compare 48).*

Blood groups are characteristics genetically determined, by a known mechanism, absolutely objective in character, absolutely unaffected by environment, not subject to mutation at any rapid rate (63, 28), and so far as we are able to discover by extensive investigation, non-adaptive. If it is possible to discover anything about the course of human evolution by classifying mankind, the most useful data should be the blood groups.

3.614 Attitude of Anthropologists Toward Blood Grouping

From a methodological point of view, the introduction by the Hirszfelds of blood group investigation into anthropology was the greatest step forward since the introduction of the cranial index by Retzius (46). One would expect the study of blood groups to occupy, as it surely will in the future, a most prominent place in all anthropological investigations. That it does not in all cases, as yet, can be attributed to three main causes: (a) the geographical variation in blood group frequencies was discovered only after other physical anthropological criteria had enjoyed extensive use; (b) many anthropologists do not know enough of the science of genetics to appreciate the advantages which blood groups offer for anthropological classification; (c) study of blood group frequencies often does not confirm ideas about "race" already held.

Some anthropologists have practically rejected blood grouping evidence. In so far as the conclusions from blood grouping confirmed their previous ideas, they have been welcomed; in so far as they have been contrary, they have been ignored, or explained away by assumptions about "mutation". Morant (42) says: " . . . the ultimate test of the value of blood-groups in particular, and of physiological characters in general, for the

* See also *Am. J. Phys. Anthropol.* **27**: 254, 1940.

particular purpose in view must demand* on whether, when sufficient data have accumulated for them, they will be found to provide reasonable results in practice." "Reasonable results" evidently means either results which agree with those of the study of morphological characters, or results agreeing with our original ideas of race, based on skin color, language, customs, etc., surely not a thoroughly scientific attitude.

Sir Arthur Keith (33) says: "A Negro or a Chinese, a Hindu or an American Indian meets us in the street, and, before he has passed, we have assessed his racial characters and made our diagnosis. We can never hope to obtain, by the use of instruments, indices, and angles, the delicacy and precision of racial diagnosis reached by the tutored and experienced eye. Travellers and explorers may become so skilled that in a cosmopolitan crowd in an eastern bazaar they will recognize the most delicate shades of race.... There are two characters which weigh with him as of especial importance: the degree to which skin, hair, and eyes are pigmented: also the hair texture."†

Most of our readers have probably observed that experienced travellers can generally spot an American on foreign streets also; the superficiality of this sort of identification of "race" is sufficiently shown by this fact. We need hardly fear that the anthropological use of blood groups will disappoint us, after this.

3.62 Data at Present Available

The amount of information on geographical variations in blood group frequencies probably exceeds that on any other physical characteristic. As a sample we give the original data of the Hirszfelds (29), who discovered the racial variation in blood group frequencies (table 60).

The Hirszfelds in some cases pooled results for persons of rather different origins. We have separated them again where possible, and have altered in some cases the designations used by them for ethnic groups.

The data available today have recently been summarized (7), and could not in any case be reproduced here. The material, as with other anthropological data, is of unequal merit. In his summary, Boyd attempts to indicate the data which were more likely to be reliable. These amount to slightly over half of the published material.

3.63 Survey of Available Data

The first point to be noted has been mentioned by Howells (31), Haldane (28), and Boyd (9). It is that, just as pigmentation and head form are more or less independent in their geographical variation, so the variation

* So in the original; "depend" must have been meant.

† From *Arabs of Central Iraq*, by Henry Field, by permission of the Field Museum of Natural History, publishers.

in the blood groups is independent of both of these. It has been stated (14), "... in (a) population sufficiently free from recent mixture to be in genetic equilibrium, no correlation is to be expected between the distributions of two characters, unless they are caused by, or affected by, the same gene." (The effect of linkage would in general be eventually masked by the effects of crossing over.)

Figure 36 shows a very schematic representation of the variation in blood groups and two other physical characters in the same part of the

TABLE 60

FIRST OBSERVATIONS OF BLOOD GROUP FREQUENCIES IN DIFFERENT ETHNIC GROUPS (MODIFIED FROM 29)

People	Number tested	Per cent of group				Frequency of gene		
		O	A	B	AB	p	q	r
English	403	44.6	44.6	7.7	3.0	0.277	0.055	0.668
French	560	43.2	42.6	11.2	3.0	.268	.081	.658
Germans and Austrians	500	40.0	43.0	12.0	5.0	.278	.088	.634
Italians (N)	201	44.8	41.8	9.9	3.5	.261	.071	.670
Italians (S)	220	46.8	36.0	14.0	3.2	.225	.096	.684
Serbs	500	38.0	41.8	15.6	4.6	.277	.116	.617
Greeks	130	38.5	42.3	16.1	3.1	.278	.118	.621
Bulgarians	500	39.0	40.6	14.2	6.2	.267	.105	.625
North Africans (Algiers)	300	39.0	37.6	18.6	4.6	.251	.134	.625
Moslems (Balkans)	500	36.8	38.0	18.6	6.6	.258	.138	.607
Russians (C)	400	41.2	33.6	21.2	4.0	.223	.148	.643
"Spanish" Jews (Balkans)	500	38.8	33.0	23.3	5.0	.224	.164	.623
Madagascans (Hova)	266	45.5	27.5	22.5	4.5	.180	.151	.674
Senegalese	238	46.2	21.0	29.8	3.0	.140	.192	.680
Annamese (Tonkin)	397	42.8	21.6	28.0	7.6	.148	.187	.654
"Hindus"	694	32.9	19.0	39.6	8.5	.150	.280	.566

N = north, S = south, C = central. According to Wiener (55), the results for Germans and Austrians were cited by memory from results of von Dungern and Hirszfeld in Germany, and of Landsteiner in Vienna.

world. It will be seen that the frequency of the gene for B can be the same in parts of Africa as in parts of Europe, in spite of the difference in pigmentation. It is probable that facts such as this have been largely responsible for the distaste many anthropologists have felt for blood grouping. On the basis of the above considerations, independent variation is not surprising.

As an illustration of this, in addition to the facts brought out by figure 36, we may point to the remarkable similarities in blood group frequencies shown in table 61, based partly on Wellisch (54).

An important consequence of these facts would seem to be that there

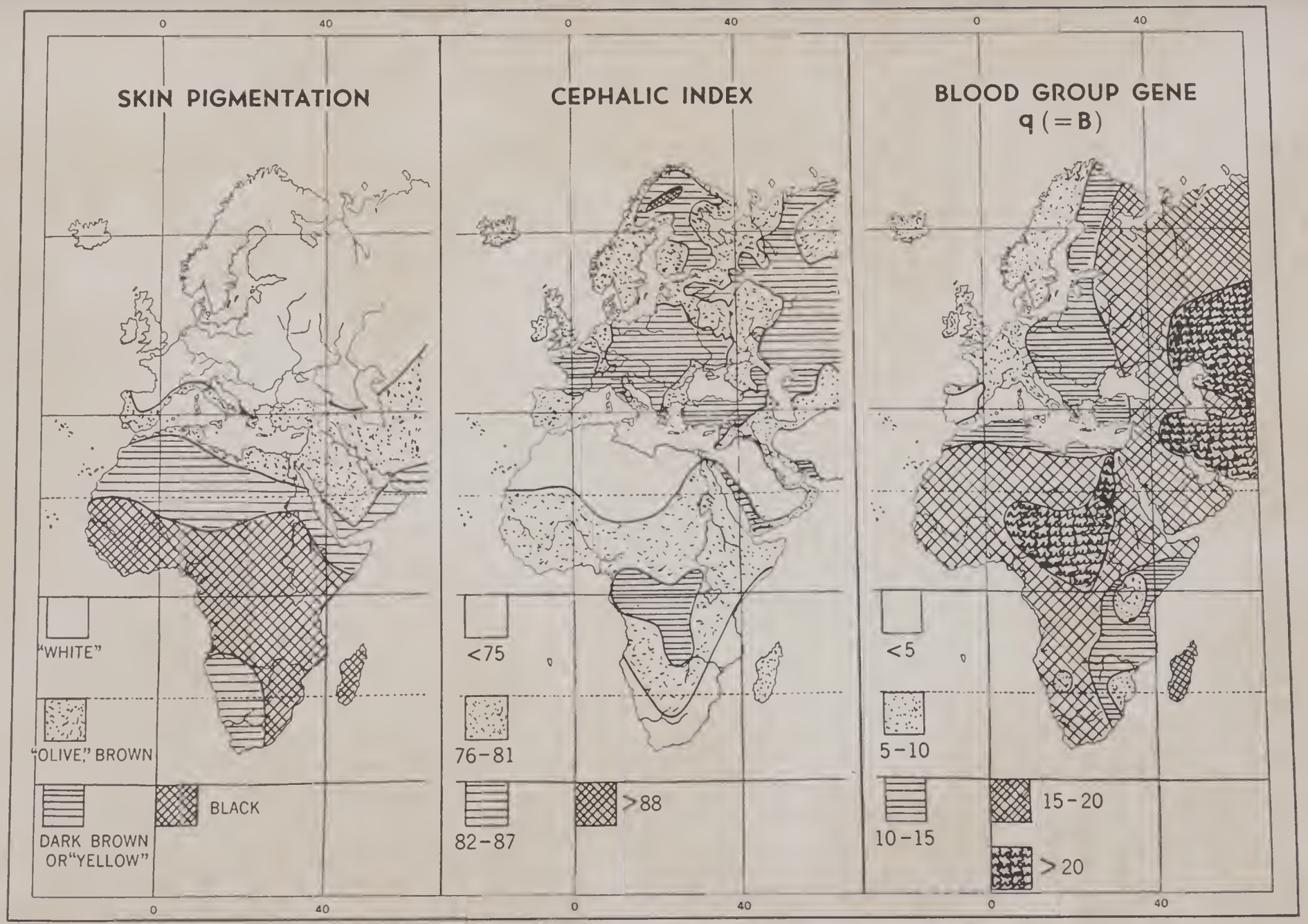

Fig. 36. Showing absence of correlation of variations in incidence of blood group B, cephalic index, and skin pigmentation (Boyd, in the *Am. J. Phys. Anthrop.*, **27**, 333, 1940).

is no particular use in basing a classification of mankind on some arbitrary combination of such characteristics. If they are in truth independent, each must be studied by itself, and we cannot hope that any given combination of characters of this sort will have any evolutionary significance. Any "race" which we distinguish on the basis of some constellation of physical characteristics will be essentially artificial, and we must not be surprised at finding all sorts of exceptions and gradations between types. As a leading geneticist has written (23), "The fundamental units of racial variability are populations and genes, not the complexes of characters which connote in the popular mind a racial distinction. Much confusion of thought could be avoided if all biologists would realize this fact.... The geography of genes, not of the average phenotypes, must be studied. Only a few attempts to apply this method in practice have been made to date. The most successful one among them is that concerning the blood groups in man."*

TABLE 61

SIMILAR BLOOD GROUP DISTRIBUTIONS IN DIFFERENT PEOPLES

People	Place	Percentage of group				Frequency of gene		
		O	A	B	AB	p	q	r
Eskimo	Labrador + Baffin Land	46.5	53.5	0	0	.318	0	.682
Aborigines	W. Australia	48.1	51.9	0	0	.306	0	.694
Cantonese	Canton	45.9	22.8	25.2	6.1	.154	.168	.678
Katangas	S. Belgian Congo	45.6	22.2	24.2	8.0	.156	.169	.675
Russians	Kazan	41.9	27.3	23.3	7.5	.194	.169	.648

Although it has certain objections, one of the most graphic methods of representing the results of blood group frequency studies in different regions is by plotting the frequency of each gene at the point corresponding to that locality, and then drawing lines of equal frequency, which we might perhaps call *isogenes*, through points where the gene frequency is the same. If enough observations are available, so that blank spaces do not appear on the map, and the data are accurate, a beautifully regular representation of the geographical trends is obtained. Figure 37, taken from a paper of Komatsu and Ito (34) shows the results of such a procedure as applied to Japan, which has been thoroughly studied in this respect.

If we attempt to apply this method to the world as a whole, we find that many of the data are unreliable, and that data do not exist for many regions, so that drawing contour lines through them gives a false impression

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Fig. 37. Variations in frequency of the A factor in Japan (Komatsu and Ito) (34)

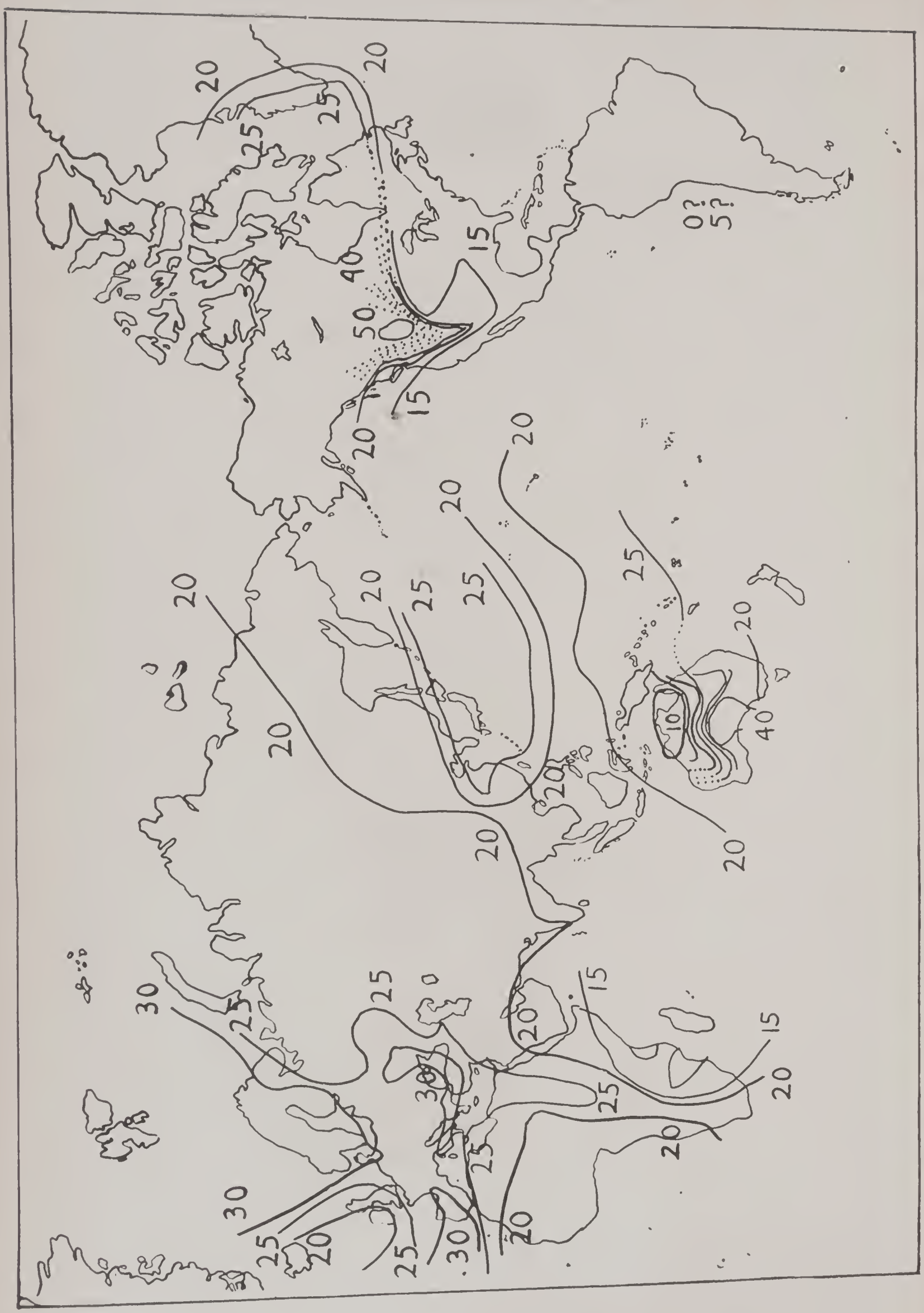

Fig. 38a

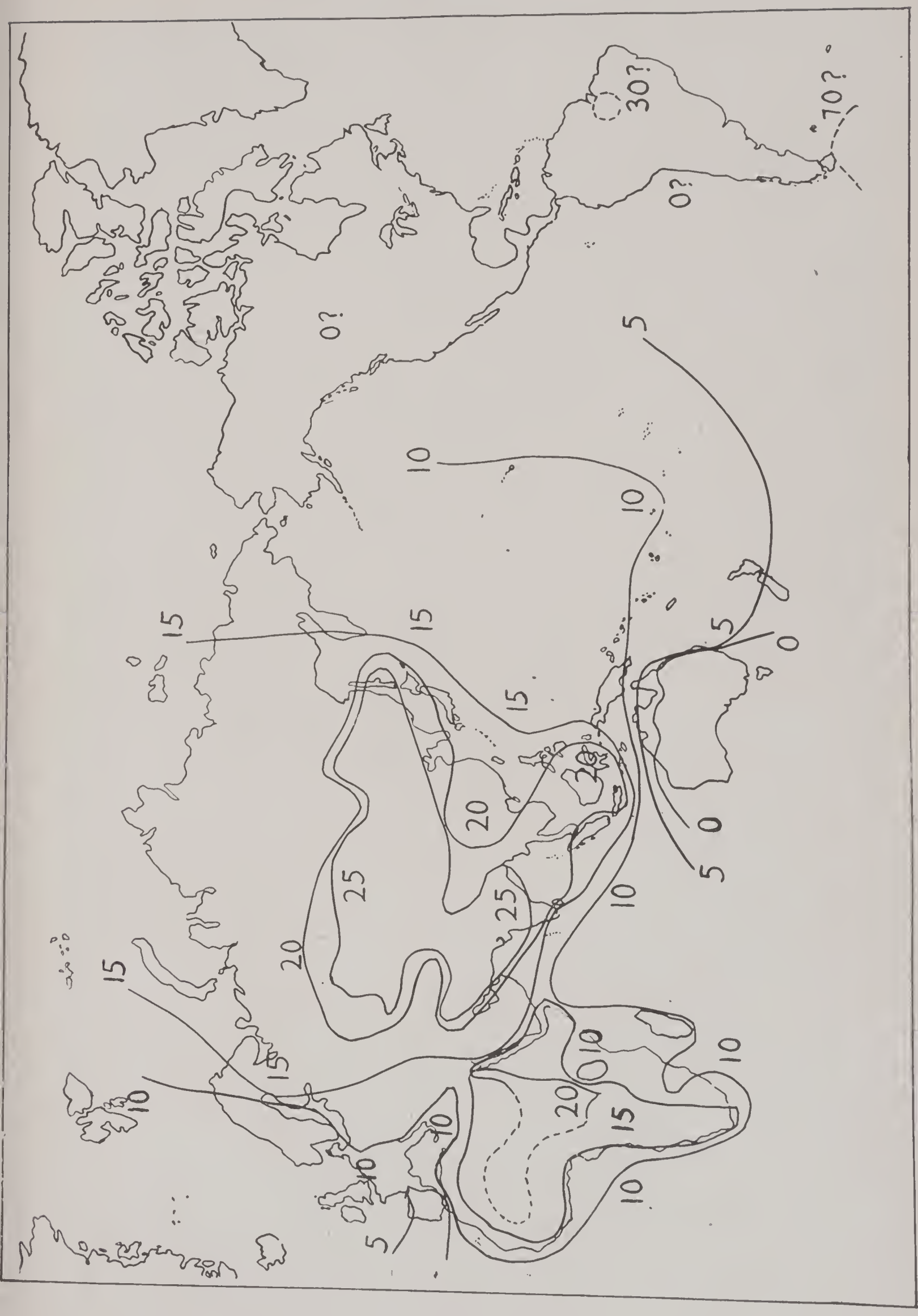

Fig. 38b

Fig. 38. Isogenes for A and B in the world: (a) blood group gene A; (b) blood group gene B.

of knowledge where there is actually ignorance. Also, the population in some regions is sparse and nomadic, so the method strictly is almost meaningless. Nevertheless, the procedure is worth undertaking as the simplest way of giving a bird's eye view of what is known. The results of the method are shown in figures 38a and 38b. (For America, Australia, and the Pacific Islands, only data on the aborigines are shown).

An inspection of these maps shows us certain things. The gene B (q) has its highest values in central Asia, with a subsidiary, slightly less high center in Africa. Aside from a few unconfirmed scattered observations, it falls off in value as we travel away from these centers, no matter in what direction. By the time we have reached America, Australia, and the farther Pacific islands, the value of q has fallen to zero. Gene A (p), on the other hand, is everywhere fairly high, save in certain American Indian tribes, and seems to reach its highest values in what we may call the outlying corners of the world ("refuge areas") as in Scandinavia, Spain, in the Eskimo, Australia, North America. Gene B seems to be concentrated around a center, and we may call it centripetal. Gene A seems to have fled from a center, and we call it centrifugal.

It will be noted that B is absent from North America, and present only sporadically in South America. A is present, in very high concentration, in some tribes in North America, but absent from others (8). It is present, though so far as we know, not quite so frequent, in South America.

A is also very high in Australia (5), but B is present only along the northern fringe, and is almost certainly a recent importation.

For details of the blood group frequencies in specific parts of the world, the reader is referred to the compilation of Boyd (7).

3.64 Interpretations of Available Data

Anthropologists and blood grouping experts are not entirely agreed as to the proper interpretation of the above facts. We shall here present an explanation offered by one of us, without intending to imply that it was necessarily correct in all details. It may at any rate indicate the sort of reasoning which may be applied.

3.641 Possible Mechanisms of Differentiation of Races

If a biologist were studying the distribution of one of the lower organisms in a certain region, and found that the frequencies of one or more of its genes showed certain geographical trends, as is the case with the genes A, B, and O, determining the blood groups in man, there are a number of ways in which he might attempt to account for his observations.

(a) He might assume that we had the result of the mixture, in various proportions, of two originally pure races. There seems to be a strong tendency on the part of some anthropologists to postulate originally "pure"

races, having certain designated complexes of characters. This is so strong that Bernstein in one of his early papers on the blood groups (2) was led to postulate the original existence of three "pure" human races, one of blood group A, one of group B, and one of O. Subsequent research has not produced any evidence that such races ever existed. Haldane (27) has pointed out that this tendency would logically involve the postulation of an originally "pure" red-headed race to account for the red-headed people in the world.

Our hypothetical biologist, in studying the gene distribution of an organism may of course postulate, not two original "pure" races, but two races both originally possessing a certain gene D, but in different frequencies. This is in general more credible, for there is every reason to believe that populations differing in the frequency of one or more genes have often been built up, and perhaps are still being built up, by the agencies of isolation, selection, and mutation.

The results of mixture of two racial groups with different frequencies of a non-adaptive gene can be calculated by simply taking an average of the two frequencies, weighted according to the relative numbers of individuals going to form the mixed group.

(b) The biologist might assume that the population under study had originally not possessed the gene D, and that D had arisen as the result of mutation. Or conversely, he might assume that the species had originally possessed 100% D, and some gene, not-D, occupying the same chromosome locus, had arisen by mutation.

It seems possible that those who first advocated the idea of mutation to explain the geographical distribution of the blood groups had the naive idea that it could be done by assuming two single mutations, one for A, and one for B. We know now that this would not suffice. A single mutation, unless it possesses definite selective advantage, will not spread in the population; worse than this, it will not even survive. It will probably die out after relatively few generations; ultimately, it is sure to do so (24). Even a gene with a 1% advantage in selection—a relatively large advantage—has in the long run only a 0.0197 probability, less than 2%, of establishing itself.

Some genes mutate repeatedly. It can be shown that to get observed values of p and q (62, 63, 28), it is requisite to assume improbably high rates of mutation, higher than any so far observed for non-lethal genes in Drosophila, in rodents, or in man. It also leads us to postulate rates of mutation so high that they ought to have been observed in the process of examining the blood groups of families; they have not. It has been shown more recently that some "facts" thought to need explanation by mutation (that American Indians are all group O) are actually not true, and need no explanation (8).

A further argument against the idea that A and B appeared in the human race as relatively late mutations is the fact that they, or substances indistinguishable from them, are found in many of the anthropoids (sec. 3.51, 3.52). Also, blood group antigens A and B have apparently been detected serologically in ancient Egyptian mummies, some of which were more than 5000 years old (13, 40), (sec. 3.65213).

(c) Since no two groups of animals are ever exposed to exactly the same environment, the biologist always considers the idea that the observed differences in the frequency of a gene are due to selection. It may perhaps be asked how it is known that blood groups are not subject to the action of natural selection; our reply must be that of course we cannot be sure that there is no selective advantage or disadvantage associated with a particular blood group gene, but so far as our evidence goes, it indicates that any such advantage or disadvantage, if it exists, cannot be very large.

The blood groups do not exhibit any tendency to be correlated with environment, and no large populations are known which do not possess at least A and O, and most peoples possess B also. The early idea that the American Indians would prove to possess only O has not been substantiated by subsequent investigation (8).

However, we must not lose sight of the possibility that the blood groups may have some selective value in regard to certain diseases, since the tests which have been made, or in fact practically any test which could be devised, would not detect small selective values. If it is possible for diseases to arise *de novo*, as was believed by at least one distinguished medical researcher (65), it might be possible that these would from time to time introduce a new direction to selection for the genes p, q, and r. But the same possibility would, of course, apply also to other physical anthropological criteria.

Agencies (b) and (c) are independent; i.e., the fact that a gene has selective advantage will not cause it to be produced any more rapidly by mutation, nor will the repeated production of a gene by mutation give it any selective value. Also of course, Lamarckism does not operate; i.e., direct modification of an organism by the environment (without selection), will not call forth genes to perpetuate this condition when the environmental stimulus has been removed.

(d) The fourth consideration of the hypothetical biologist is isolation which, as Gates says (25), "... must have been a factor of extraordinary importance in the early history of man."

Wright has shown mathematically (58, 59, 60, 61) that even in populations which we might not at first sight consider small, the chance fluctuations in gene frequencies with succeeding generations will often result in a gene's being lost. According to Dobzhansky (23), " ... the differentia-

tion of a species into local or other races may take place without the action of natural selection. A subdivision of the species into isolated populations, plus time to allow a sufficient number of generations to elapse (the number of generations being a function of the population size), is all that is necessary for race formation."*

(c) A concept which is probably used by anthropologists more often than by zoologists is that of inbreeding. In the proper sense this means matings among close relatives. It is sometimes suggested that the differences found between a certain human group and its neighbors are due to inbreeding (45). Mathematical treatment of the problem shows, however, that the importance of inbreeding has probably been overestimated.

Inbreeding is of course powerless, without selection, to increase the original frequency (in the detached population) of the gene. The usual effect of inbreeding (47) is to increase the proportion of the homozygotes, and decrease the heterozygotes. If inbreeding is allowed to go on long enough, the types of homozygous individuals appear in the same proportions as were their types of gametes in the original population.

Wright (56, 57) and Hogben (30) have derived formulas from which it may be calculated that (cf. 8) even the maximal change produced by inbreeding in the frequency of genetic characters is rather less than might have been expected, and inbreeding will certainly never produce a "pure" from a "mixed" race. Even this change is dependent upon continued inbreeding, and can be completely abolished by one generation of random mating (22). Bernstein proposed for the value,

$$1 - \left[\frac{MN}{2} \Big/ \left(M + \frac{MN}{2}\right)\left(N + \frac{MN}{2}\right)\right]$$

which would measure the deviation from the expected of the proportion of heterozygotes in the case of the M, N types, the term "coefficient of inbreeding" (Inzuchtsgrad), (cf. 48a). Boyd and Boyd (16) found no significant effect on blood group and type frequencies in a population known to be highly inbred.

3.642 Information on Early History of Man, Provided by Blood Group Studies

Purely as an illustration of the application of these principles to an actual case, we may offer some speculations based on our present knowledge of blood group distributions.

If our knowledge of the behavior of man today is any guide, we can safely assume that our ancestors, evidently small in numbers, readily

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formed isolated groups, probably in many cases small enough for Wright's mechanism of gene fixation to operate. It seems probable that most of the diversity in non-adaptive characters in man goes back to this period, which in the case of the American Indians, may even have continued into the Christian era. The geographical distribution of such "fixed" genes would be entirely accidental.

If we are convinced that the blood groups are non-adaptive, and are derived from the A and B of our anthropoid ancestors (assuming they, like the present-day anthropoids, possessed them), then we could suppose that the present world distribution of the blood groups is largely the result of mechanism (d), isolation, followed by the operation of mechanism (a), mixture.

The present-day distribution of blood groups suggests that in some of these early groups only genes A and O were present. It would seem likely that western Europe, Australia, and probably North America were populated originally by the descendants of peoples which had lost the gene B, but possessed A and O, with p and r perhaps in about the proportion of 3 to 7.

Since A is quite generally more frequent than B today, we may suppose it was so in the original human population. It will be understood, therefore, why B could be lost more readily than A by the process of isolation. If B has been lost several times in isolated groups, we need not be surprised at lack of resemblance in other features shown by peoples lacking B.

Whatever the exact sequence of migrations to the New World from Asia, Boyd considers it almost certain that isolation, in Wright's sense, has been largely responsible for the characteristic "patchy" blood group picture in America (9).

Haldane (28) believes that we are justified in supposing that "Neolithic Europe was occupied by peoples of a blood group distribution not unlike that of American tribes, that is to say with B rare or absent (q small or zero) whilst the frequencies of O and A (and therefore the values of r and p) were variable. . . ." He supposes further that "the areas with high p were however rather irregularly distributed. There was probably one around the Baltic, and another in the Iberian peninsula. . . . This primitive European population has been diluted by immigration from the east of peoples in whom q was higher and r lower, whilst p lay (in most cases at least) within the European range."

3.643 Hypothetical Early Migrations

This picture is consistent with the idea that earliest man probably possessed a blood group distribution not far different from that of present day Asiatics or Negroes, say $p = 0.25$, $q = 0.15$, $r = 0.60$, but that isolation early produced groups devoid of gene B. After a possible small

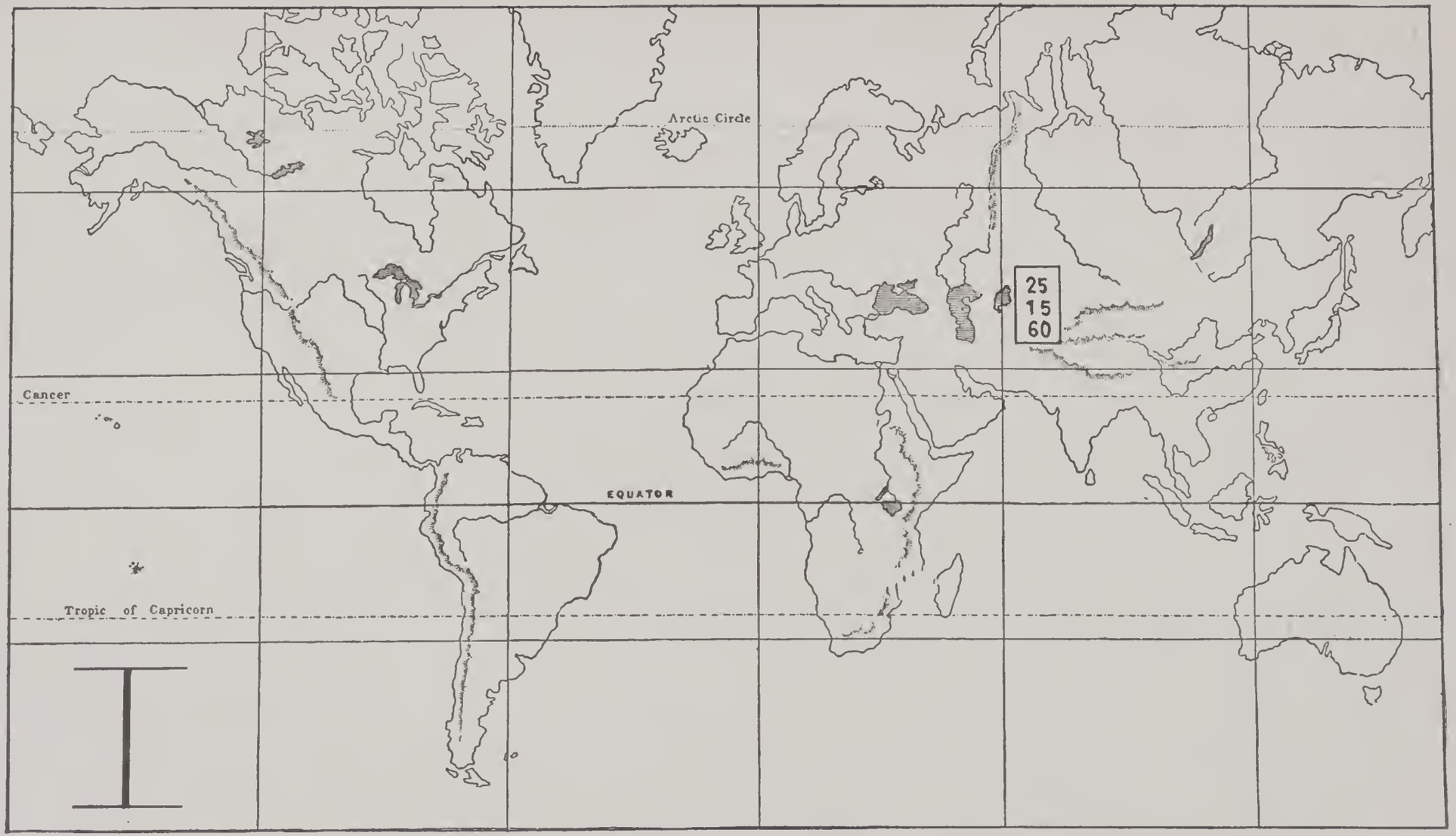

Fig. 39a

Fig. 39. Four hypothetical stages of dispersion of early human populations (Boyd, in the *Am. J. Phys. Anthrop.*, **27**, 333, 1940).

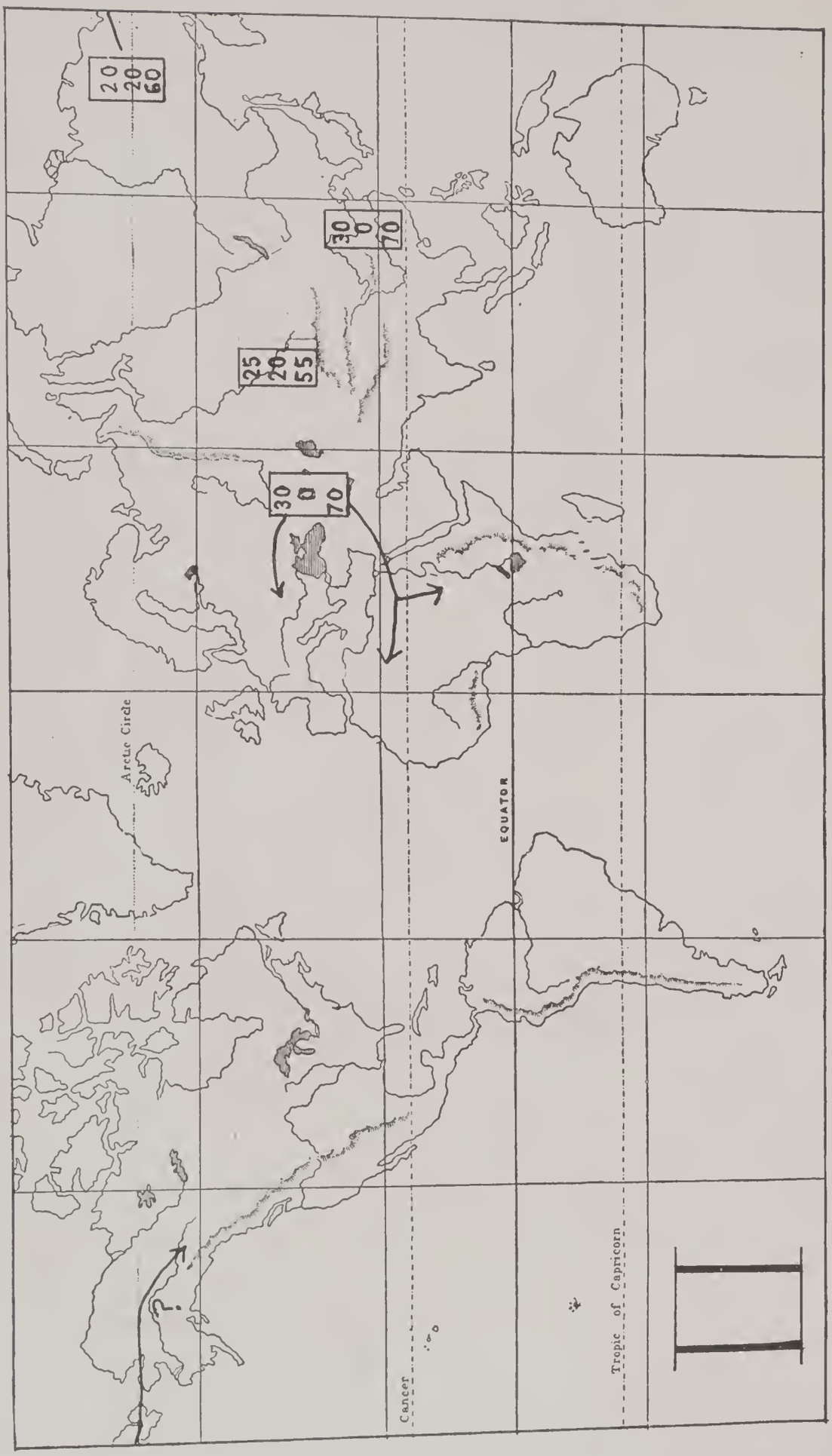
20
20
60
30
0
70
25
20
55
30
0
70
Arctic Circle
EQUATOR
Cancer
Tropic of Capricorn

Fig. 39b

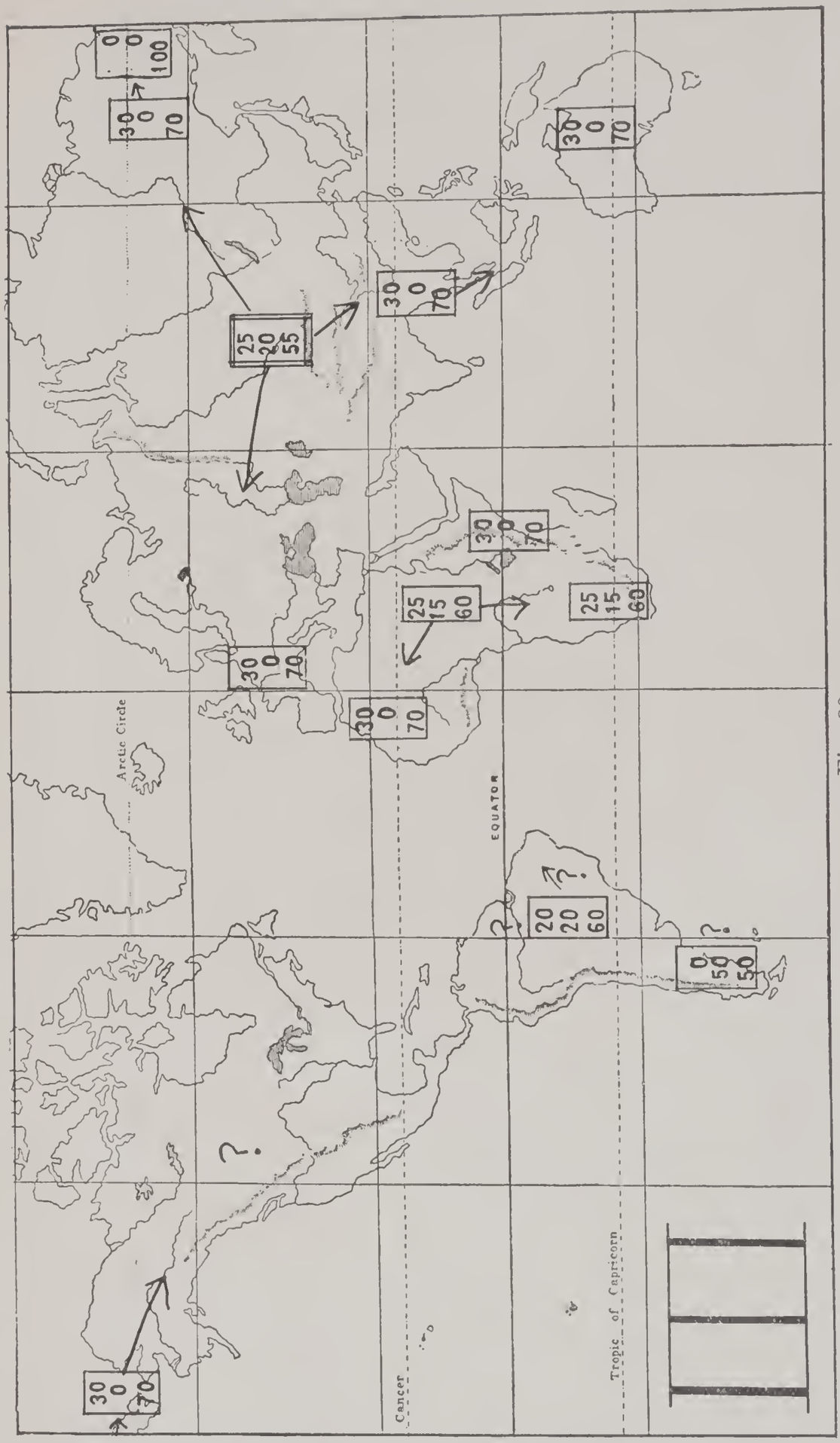
Arctic Circle
EQUATOR
Cancer
Tropic of Capricorn
0 0 100
30 0 70
25 20 55
30 0 70
30 0 70
30 0 70
25 15 60
25 15 60
30 0 70
30 0 70
20 20 60
0 50 50
30 0 70
?
?
?
?

Fig. 39c

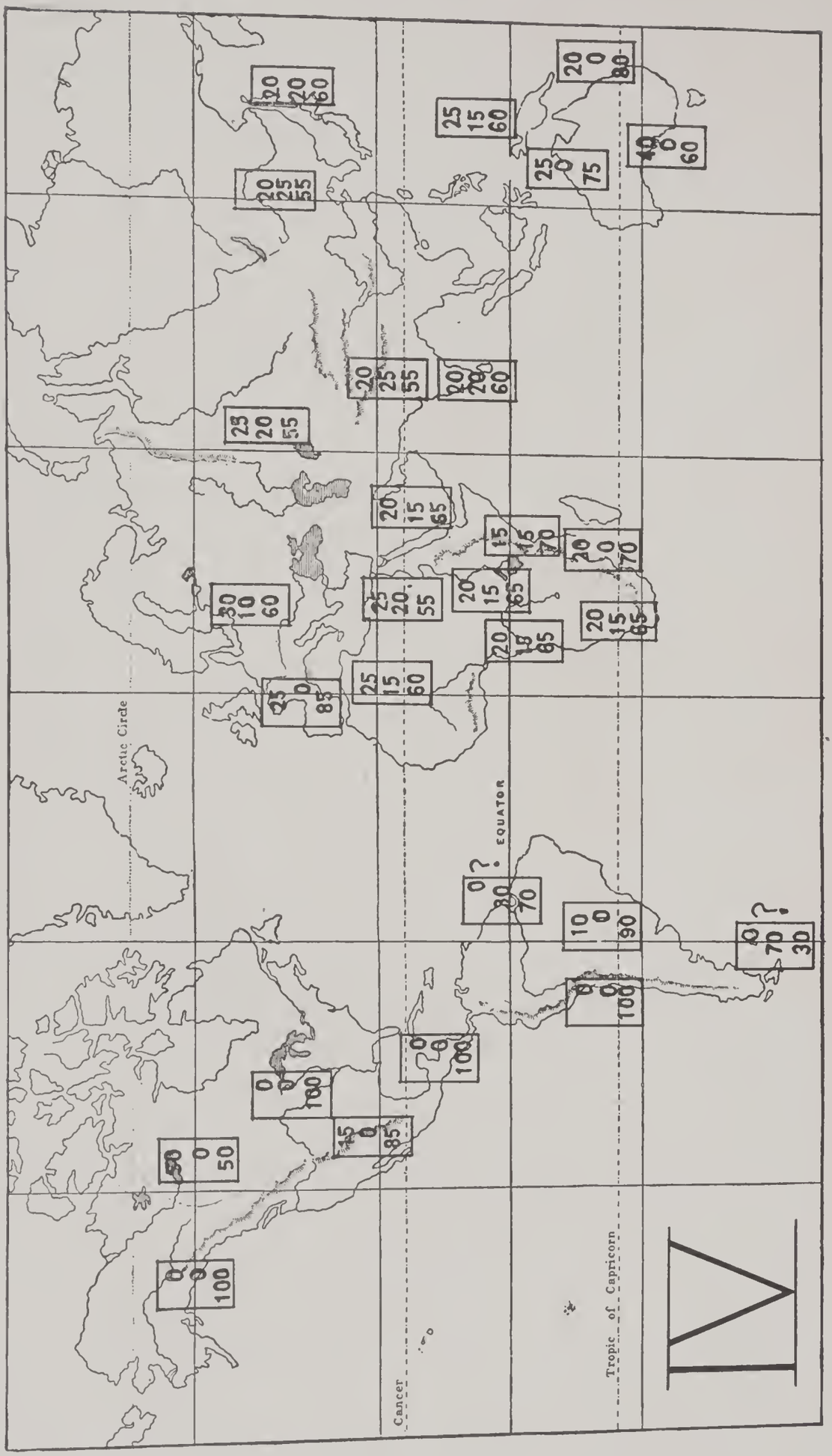

Fig. 39d

dribble of people with some B (there is some evidence from serological studies on American Indian mummies that B was present in some prehistoric Indians (63)), such groups without B later entered America, probably in more than one wave, and isolation in the new environment was favored to such a degree that still further differentiation took place, giving some tribes with much A, some with a moderate amount and some, probably, with none. In central Asia, and in Africa, groups with about the normal frequencies would seem to have remained. All this may be supposed to have taken place some time before or during the Pleistocene.

Then followed a great numerical increase of the populations in Asia, and to some extent in Africa. These spilled over somewhat into Europe, modifying the blood group distribution there, and leaving the east-to-west gradient of q seen on the map. To a certain extent, doubtless, the A possessors retreated, to "refuge areas", before the advance of the B possessors, whom we may perhaps suppose to have been more advanced in the arts of war, hunting, and/or agriculture. The Asiatic B possessors did not penetrate, in this last advance, to Australia or the New World, though they did reach, for instance, New Guinea.

Four possible stages in the hypothetical distribution of early man and the blood groups are shown in Figure 39.

The intervals between the stages are supposed to be very long. Changes from one stage to the other need not have been simultaneous; in particular, events in America may have been even later than other changes shown in stage IV. Man is shown losing B before entering the New World, but it is equally possible that this happened after reaching America.

While these early human groups were expanding and gradually penetrating outlying areas such as Europe, the process of human evolution was going on in other respects. The adaptive characters, such as pigmentation, were being modified under the influence of selection. In northern Europe man was losing eye, skin and hair pigmentation. In tropical climates he was acquiring deeper pigmentation. The shift of human populations since this process got under way has introduced exceptions to the correlation between climate and pigmentation, but has not obscured the general relation.

3.644 The M and N Factors

The frequencies of these blood types in populations have not been studied to nearly the extent that A and B have.

Since the M, N types are determined by a single pair of genes, it is sufficient to study the frequency of one of the genes only, as the sum of the frequencies of the two must equal unity. A very rough outline of our present knowledge of the frequency of the gene M is shown in figure 40.

From the above discussion it is clear that the creation by isolation of

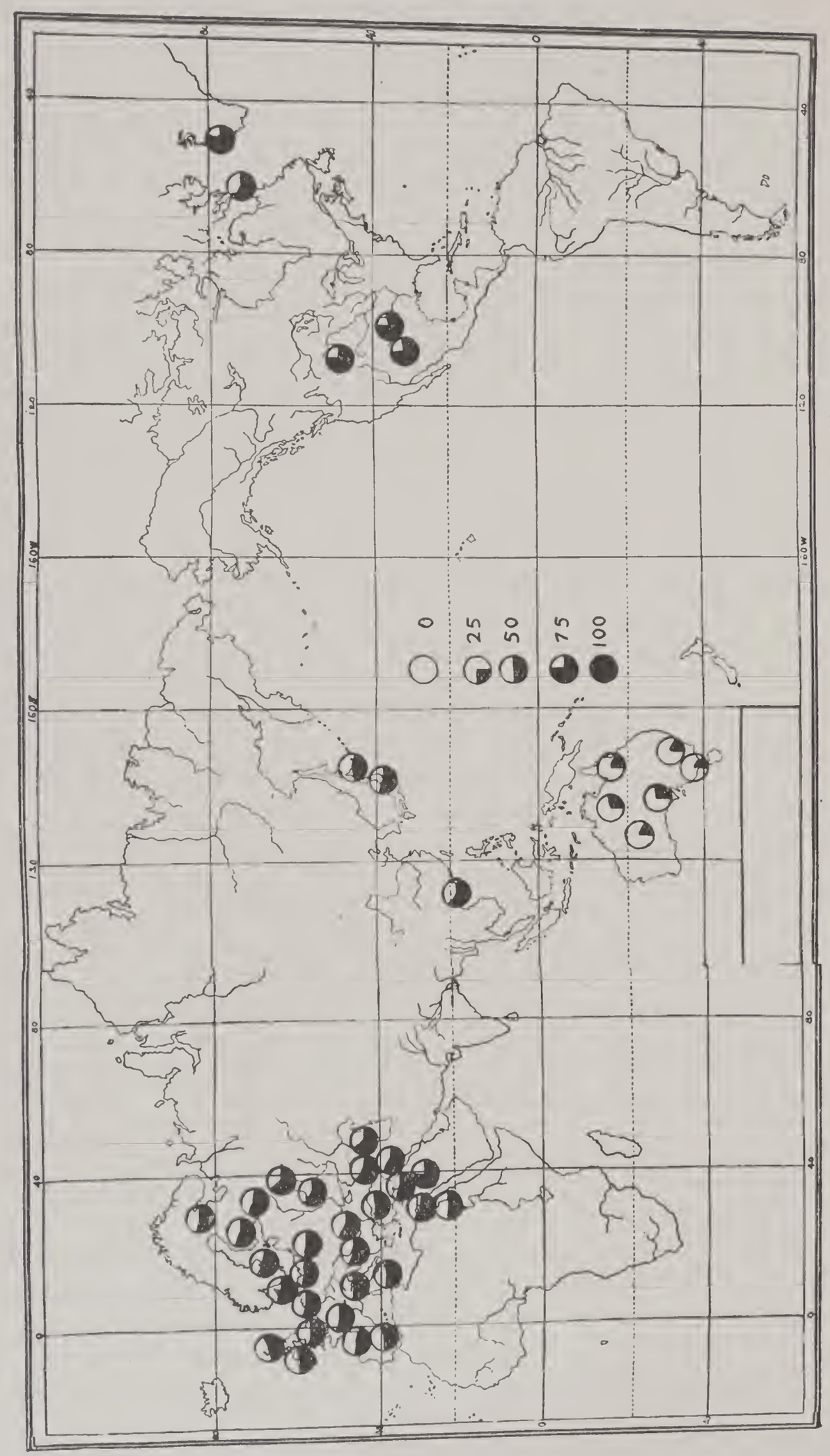

Fig. 40. Frequency of M gene in various parts of the world

small ethnic groups differing in gene frequencies would also apply to other genetically determined characters, but we would not expect that there would be any correlation between the frequencies of independent genes. A certain gene D may be lost from population x, while in population y it is retained. Population y may lose the gene F. Occasionally a population may lose both. The resulting geographical distribution of D may be quite different from that of F. As we have already pointed out, pigmentation and cranial index seem to be distributed independently of the blood groups.

There seems to be evidence of the same tendency in the meager data we possess on the distribution of the genes determining M and N. The North American Indians, insofar as they have been tested, have a uniformly low percentage of gene N. This is independent of whether or not they possess high per cents of A. The Rwala Bedouin, and possibly a number of Bedouin tribes, also have low values of N, but have considerable A and B (15, 32).

Birdsell (5) found that the contrary is true of the Australian aborigines. There M is very low, in one instance so low that it seems possible that the group in question may actually have lost the gene completely at one time.

The general picture is shown in figure 40.

It is probably significant that the gene most often rare or absent in human populations is B, if we assume that the primitive population started with a value of q of only 0.15 or so. Genes A, N, and M, starting presumably at higher values, have been lost less often, the first two perhaps only in America, the last only in Australia. Whether selection must also be invoked to account for this is still a question.

3.645 Other Useful Criteria

It seems probable that the physical anthropology of the future, if the science continues to advance, will be based largely on the study of characters of the sort discussed. This does not restrict us to blood groups, however. It is clearly desirable that excitement over the results achieved up to the present should not lead to an exclusive application of serological methods. There is no particular magic in the blood, and it goes without saying that a comprehensive science of anthropology will not be based on a single criterion. It is to be hoped that the development of genetics will bring to light other useful characteristics which will have the same advantages as the blood groups. Other characters which may acquire importance are direction of the hair whorl, pattern of papillar lines on fingers and toes, the "secreting" factor of Schiff and Sasaki (sec. 1.43), and possibly certain genes influencing hair form, and certain other inherited properties of the blood. This brief list will certainly be extended in the future.

We should guard against false optimism, which has once before led to a reaction against the use of blood groups in anthropology, when it was found that, contrary to what was expected at first, it was not possible to separate, by means of blood groups, mankind into well defined races. We are still under the influence of this reaction.

It may never be possible to reconstruct the course of human evolution to the extent we should like. Even if adequate skeletal remains should be eventually discovered, so that we were able to construct morphological lines of descent for all important racial groups, and to carry them back to the points where they branched off from the original stock, it is hard to see how this can tell us anything about the evolution of pigmentation, or hair form, or blood groups, or artistic ability, since we know of no genes affecting at the same time the skeletal characteristics and these characters. And the conclusions we can draw from a study of present-day man will always be rather limited.

3.65 Technic

3.651 Investigations of the Living

3.6511 Sources of Material. It is practically impossible to investigate all the individuals of a whole group. Therefore it is necessary to select a sample which will represent as well as may be the entire population. Frequently this condition is satisfied by testing school children. Studies on soldiers give, as a rule, a good picture of the population, though here the sample is no longer strictly a random one, for a selection, probably without effect on blood group frequencies, has already been made, for example, in regard to age and health.

A possibly more serious selection has already been made when the subjects are inmates of asylums, hospitals, prisons, and members of special professions. Although it is often necessary to make use of such material, one must keep in mind possible effects of the selected nature of the material. No certain proof has ever been offered that the incidence of any disease is significantly different in different blood groups, but the possibility should not be lost sight of, even when the source of material is simply bloods sent in for Wassermann tests.

The investigator will not always be able to choose the material he would prefer, but must in many cases make use of what he can obtain. It is usually easiest to get permission to test patients in hospitals, and inmates of prisons; the bloods can be taken very rapidly under such conditions. It is very difficult in some countries to get permission to test school children. In the case of primitive and nomadic peoples, it may be necessary to seek them out in their native habitat. In such cases it is often wise to interest one of the local chiefs financially in the work, so as to improve the cooperation. The choice of an interpreter, if the investigator does not speak

the language fluently, is a matter of great importance, and a wrong choice may practically defeat the purpose of the expedition.

Records kept should be such that any questions which might arise later as to the origin or composition of the human material could be answered. The name of the subject, his age, sex, and place of origin should be recorded. Information as to the place of birth of his parents may also be useful. Religion should be recorded because of possible correlation with racial origin. In hospitals and laboratories a record of the patient's disease should be made if only to ascertain later that certain diseases do not alter appreciably the normal frequencies.

When possible, the M, N types, as well as the A, B groups, should be investigated.

3.6512 Requirements for the Technic. Since all results will depend ultimately on a series of tests of individuals, the question is: how, most easily, to test a large number of blood samples from different persons.

In contrast to blood group determinations for surgical and medico-legal purposes, here *absolute* accuracy is not required. It will make no difference if one or two errors are made in several hundred tests, especially as errors would probably be first in one direction, then in another, and should thus largely cancel, so the determination may be simplified by omission of most of the otherwise necessary controls. If suitable sera are used, few if any errors will be made, even in a large series. Tests on the individuals' sera are not needed; it is sufficient to group the cells alone. For the A, B tests only two test tubes are thus needed for each blood.

Dispensing with controls means that it is all the more necessary to make certain each day of the suitability of the test sera. It is necessary to be sure that the sera used, first, belong to the proper groups, second, that they are strong enough to react clearly even with weakly sensitive cells in the time allowed for the test. This is best accomplished by including bloods from persons of known group, say members of the expedition, with the tests each day. It is permissible to use as testing fluids mixtures of several strong sera of the same group. Absorbed immune sera (sec. 2.3) have been found very satisfactory for work on expeditions (14, 15).

It goes without saying that any investigator who is going to apply any experimental technic, without full controls, will first convince himself of his own personal competence to do the tests, by carrying out a large series of determinations with careful retests, or with independent checks by another worker; if bloods of known group are available they should be included in the series. This requirement applies *ex post facto* when an investigator has found particularly striking results which differ from those previously observed. Of the results published up to June, 1938, over ten per cent, on statistical test, show internal evidence suggestive of error (7).

3.6513 Procedure. Blood is taken, as described above (sec. 2.241), from the finger or the ear lobe, and one or two large drops placed directly in 1.0 to 1.5 cc. of diluting fluid.

Diluting fluid. Dissolve 0.9 g. NaCl in 100 cc. distilled water. (To this 0.5 g. sodium citrate may be added if desired, but it is probably unnecessary.) If tap water *must* be used, a few control bloods should be tested to check its suitability.

The tests (sec. 2.251). The test tubes are placed in racks designed for this purpose (figure 5). Wire racks holding 4 rows of 12 each are satisfactory. Large wooden blocks with rows of holes are convenient and cheap, but somewhat heavier and bulkier than the wire racks. Halber and Mydlarski proposed a specially designed holder for 400 tubes, suitable for transportation (26).

The tests are carried out on the day the blood is taken, if possible. If the weather is very cool, or if refrigeration is available, they may be done the following day.

If the A, B groups alone are being determined, the test tubes are arranged in two rows; in each tube of the first row there is placed 0.1 cc. (2 drops) of test serum A(anti-B), in the farther row 0.1 cc. (2 drops) of test serum B(anti-A). (If it is known that the test sera are fresh and react strongly, as is often the case with immune sera, they may be diluted 1:2 or 1:4 before use, for the sake of economy, or one drop of serum and one of saline may be used.) Each pair of tubes, one standing behind the other, serves for the tests on one individual's blood. To each tube of the pair there is added 0.05 cc. (1 drop) of the corpuscle suspension.

After everything has been added, the tubes are thoroughly shaken and allowed to stand at room temperature. With strong immune sera the reading can be made after 15 to 30 minutes; with human test sera at least an hour, preferably two or three hours, should be allowed. While one rack of tubes is standing, other tests may be set up. Before reading, the tubes are gently shaken. The reading is done with the naked eye. If it is wished to detect all A_2 and A_3 bloods, the negative bloods may be examined with a hand lens. (Compare figures 5 and 10.)

3.6514 Evaluation of the Results. Certain elementary principles of statistics should be kept in mind in assessing the significance of the results, and in comparing them with the observations of others. To have any confidence in group frequencies, it is of course necessary to feel that the individual observations are reliable. One must know that the investigator avoided the danger of pseudo-agglutination, and particularly that he used sufficiently strong test sera. Too weak sera cause errors in a definite direction (53).

3.65141 Comparison of Different Series. The size of the sample must also be considered. The standard deviation of the percentage values obtained may be simply calculated, or found in tables in various books (see table 62). We may use this to test roughly if an observed difference is significant. More accurate evaluation of the significance of difference between two series may be had by other methods, such as the χ^2 test (51, 64).

TABLE 62

STANDARD DEVIATIONS (σ) OF BLOOD GROUP FREQUENCIES (PER CENT)

Size of sample	Observed per cent				
	10 or 90	20 or 80	30 or 70	40 or 60	50
100	3.00	4.00	4.58	4.90	5.00
150	2.45	3.27	3.74	4.00	4.08
200	2.12	2.83	3.24	3.46	3.54
250	1.90	2.53	2.90	3.10	3.16
300	1.73	2.31	2.65	2.83	2.89
400	1.50	2.00	2.29	2.45	2.50
500	1.34	1.79	2.05	2.19	2.24
750	1.10	1.46	1.67	1.79	1.83
1000	0.95	1.27	1.45	1.55	1.58
1500	0.78	1.03	1.18	1.27	1.29
2000	0.67	0.89	1.03	1.10	1.12
3000	0.55	0.73	0.84	0.89	0.91
5000	0.42	0.57	0.65	0.69	0.71
10,000	0.30	0.40	0.46	0.49	0.50
20,000	0.21	0.28	0.32	0.35	0.35

Use of table. Suppose we have two samples of a population, each of 100 individuals. In one the per cent of group O has been found to be 40%, in the other 50%. May we assume a real serological difference in the two samples? From the table, σ for 40% and 100 individuals = 4.90; for 50% and 100 individuals we find 5.00. We obtain σ_D, the standard deviation of the difference, from the relation $\sigma_D = \sqrt{\sigma_1^2 + \sigma_2^2}$. We fine $\sigma_D = 6.85$. The difference, 10, between the two observations, divided by $\sigma_D = 1.46$. Since this is less than the ratio 2.00, which is arbitrarily taken as the boundary between significance and non-significance, the observations cannot be said to differ significantly. If the samples had been twice as large, we should obtain a ratio D/σ_D of about 2.2, which is by agreement significant, but not strikingly so.

Of course no amount of statistical calculations can be counted on always to protect us from false conclusions due to erroneous determinations. They may even at times create a false picture of certainty which is not justified. If an investigator, using an unsatisfactory A serum, should find a percentage of group O of 70, while a more careful worker found for another sample of the same population a percentage of O of 40, the difference between the two series would appear mathematically sig-

nificant, even though the samples might be small. And although the data obtained by the first worker might even be compatible with the conditions of genetic equilibrium (sec. 3.65143), suggesting an anthropological difference between the two samples, the real cause of the discrepancy would be faulty technic.

TABLE 63

CALCULATION OF p, q, AND r FOR DIFFERENT BLOOD GROUP DISTRIBUTION

x	$\sqrt{x}$	Δ	$1-\sqrt{1-x}$	Δ	x	$\sqrt{x}$	Δ	$1-\sqrt{1-x}$	Δ
.01	.100		.005		.26	.510		.140	
		41		5			10		6
.02	.141		.010		.27	.520		.146	
		32		5			9		5
.03	.173		.015		.28	.529		.151	
		27		5			10		6
.04	.200		.020		.29	.539		.157	
		24		5			9		6
.05	.224		.025		.30	.548		.163	
		21		5			9		6
.06	.245		.030		.31	.557		.169	
		20		6			9		6
.07	.265		.036		.32	.566		.175	
		18		5			8		6
.08	.283		.041		.33	.574		.181	
		17		5			9		7
.09	.300		.046		.34	.583		.188	
		16		5			9		6
.10	.316		.051		.35	.592		.194	
		16		5			8		6
.11	.332		.057		.36	.600		.200	
		14		5			8		6
.12	.346		.062		.37	.608		.206	
		15		5			8		7
.13	.361		.067		.38	.616		.213	
		13		6			8		6
.14	.374		.073		.39	.624		.219	
		13		5			8		6
.15	.387		.078		.40	.632		.225	
		13		5			8		7
.16	.400		.083		.41	.640		.232	
		12		6			8		6
.17	.412		.089		.42	.648		.238	
		12		5			8		7
.18	.424		.094		.43	.656		.245	
		12		6			7		7
.19	.436		.100		.44	.663		.252	
		11		6			8		6
.20	.447		.106		.45	.671		.258	
		11		5			7		7
.21	.458		.111		.46	.678		.265	
		11		6			8		7
.22	.469		.117		.47	.686		.272	
		11		6			7		7
.23	.480		.123		.48	.693		.279	
		10		5			7		7
.24	.490		.128		.49	.700		.286	
		10		6			7		7
.25	.500		.134		.50	.707		.293	
		10		6					

Use of table: Suppose O = 38%, A = 41%, B = 15%, AB = 6%. Then x = 0.38, $r = \sqrt{x} = 0.616$. Since $\bar{O} + \bar{B} = 1.00 - (\bar{A} + \bar{A}\bar{B})$, and $\bar{A} + \bar{A}\bar{B} = 0.47$, we find p, in the fourth column, = 0.272. Similarly, $\bar{O} + \bar{A} = 1.00 - (\bar{B} + \bar{A}\bar{B})$, = 1.00 − 0.21, we find q = 0.111. If the first decimal of the blood group percents is given (there is seldom any use in having more than one decimal place), the values of p, q, and r are obtained by interpolation, using the differences listed under Δ.

3.65142 Calculations of Gene Frequencies. Different percentages of a given blood group are strictly not directly comparable one with the other unless they are of the same order of magnitude, but calculated gene frequencies may always be compared. For example, if one population has a percentage of group A of 10, and another a percentage of 25, it is inexact

to assume a difference in A content of 15 per cent between the two. The excess of A in the 25% population is really higher, for such a population

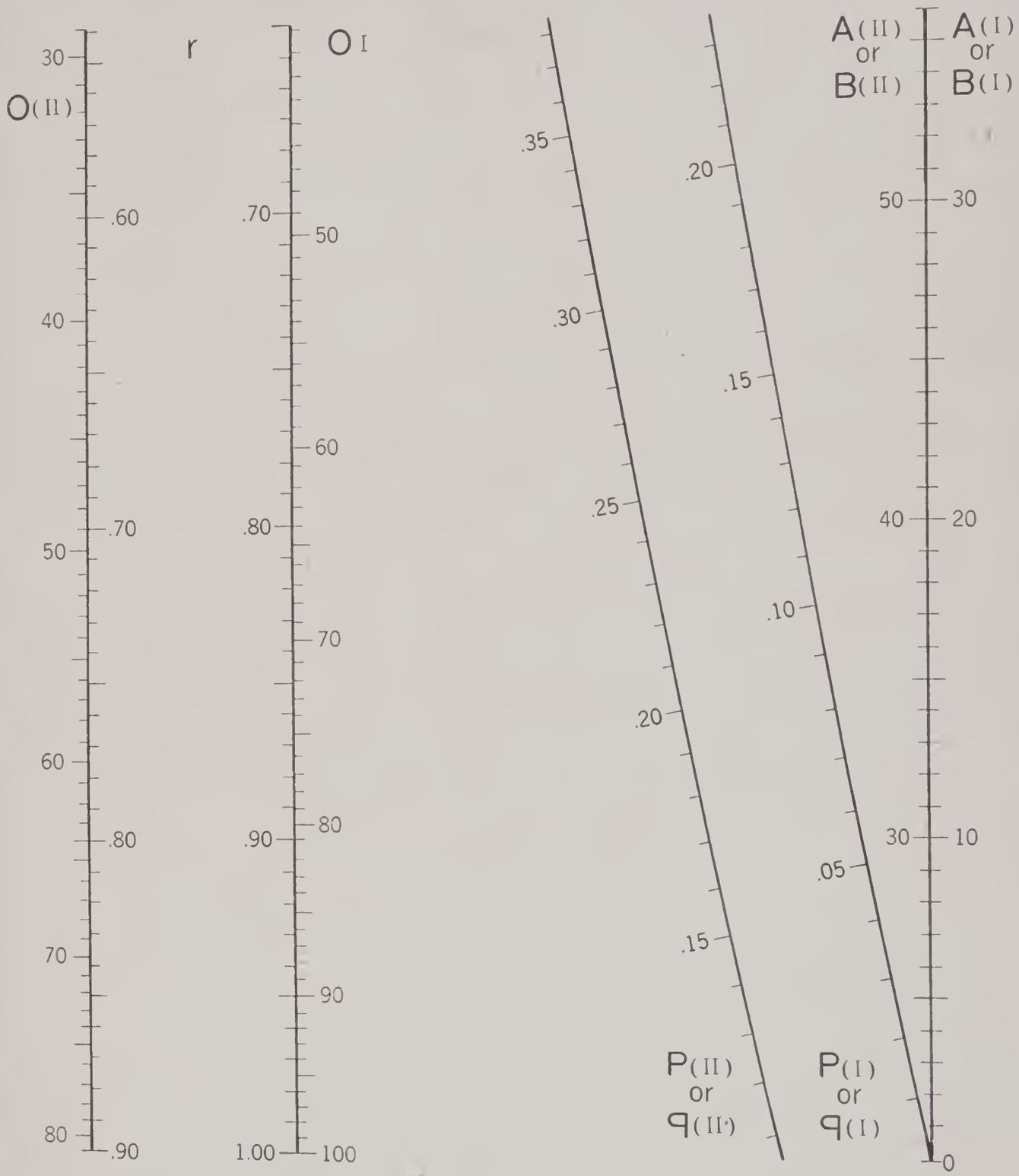

Fig. 41. Nomogram for calculating frequency of blood group genes. Index line set to values of O and A (or B) on scales O I and A I, or on O II and A II; p (or q) is read on scale p I or p II respectively. The value of r is read opposite the O value.

contains more homozygotes for A (i.e. persons possessing *two* A genes). Therefore it is in general desirable to base comparisons on gene frequencies.

Bernstein designated the frequencies of the genes O, A, and B in the population by the letters r, p, and q respectively. Thus, the various blood group frequencies in different peoples can all be reduced to variations in

the proportions of three genes. These gene frequencies may be calculated for populations in genetic equilibrium by the formulas: $r = \sqrt{\bar{O}}$, $p = 1 - \sqrt{\bar{O} + \bar{B}}$, $q = 1 - \sqrt{\bar{O} + \bar{A}}$, or, $r = \sqrt{\bar{O}}$, $p = \sqrt{\bar{O} + \bar{A}} - \sqrt{\bar{O}}$, $q = \sqrt{\bar{O} + \bar{B}} - \sqrt{\bar{O}}$. The symbols $\bar{O}$, $\bar{A}$, and $\bar{B}$ represent the *frequencies* (per cent divided by 100) of the respective groups in the population.

In the ideal case, the two methods of calculating p and q will give identical results. For most actual data, there will be a slight difference. The difference, $1 - (p + q + r)$, which we use as a measure of the departure of the figures from the relations required by genetical equilibrium according to the Bernstein theory, will have the same absolute value in either case, with only a difference in sign. There is no difference in the amount of labor involved in the calculations by the two methods. As an aid to calculating by the first method, table 63 may be found helpful (3).

Formulas for making the best statistical estimates of the gene frequencies have been proposed by Stevens (50). For rapid calculations, nomograms have been proposed by Konorski (35), and Boyd (6). (See figure 41.) Compare also Bernstein (3, 4).

3.65143 Statistical Test. For populations in genetic equilibrium the sum of p, q, and r will be close to 1, but even if no errors are made there may be discrepancies due to variations in random sampling. It may be tested whether the sum actually found is significantly different from 1 by calculating the standard deviation of the difference (Bernstein) and dividing. The standard deviation, σ_D, of the difference $1 - (p + q + r)$ is given by

$$\sigma_D = \sqrt{pq/2V(1 - p)(1 - q)}$$

where V is the number of persons examined. This computation can also be made by a nomogram (6) (figure 42).

Theoretically, the quotient D/σ should not exceed 2 where the variations are due to chance, if we follow the usual convention. Figures calculated from determinations of known reliability give on the whole results agreeing satisfactorily with this expectation. In the case of larger deviations, error in the determinations, or lack of genetic equilibrium in the population, or both, are suggested. The former would be expected to give values of $p + q + r$ (calculated from Bernstein's formulas) less than 1; the latter, values greater than 1. The nomogram given here would give values of p, q, and r such that the sign of the difference from 1 is reversed.

Comparison of the values of p, q, and r found for different ethnic groups may be made by the graphical representation proposed by Streng (52).

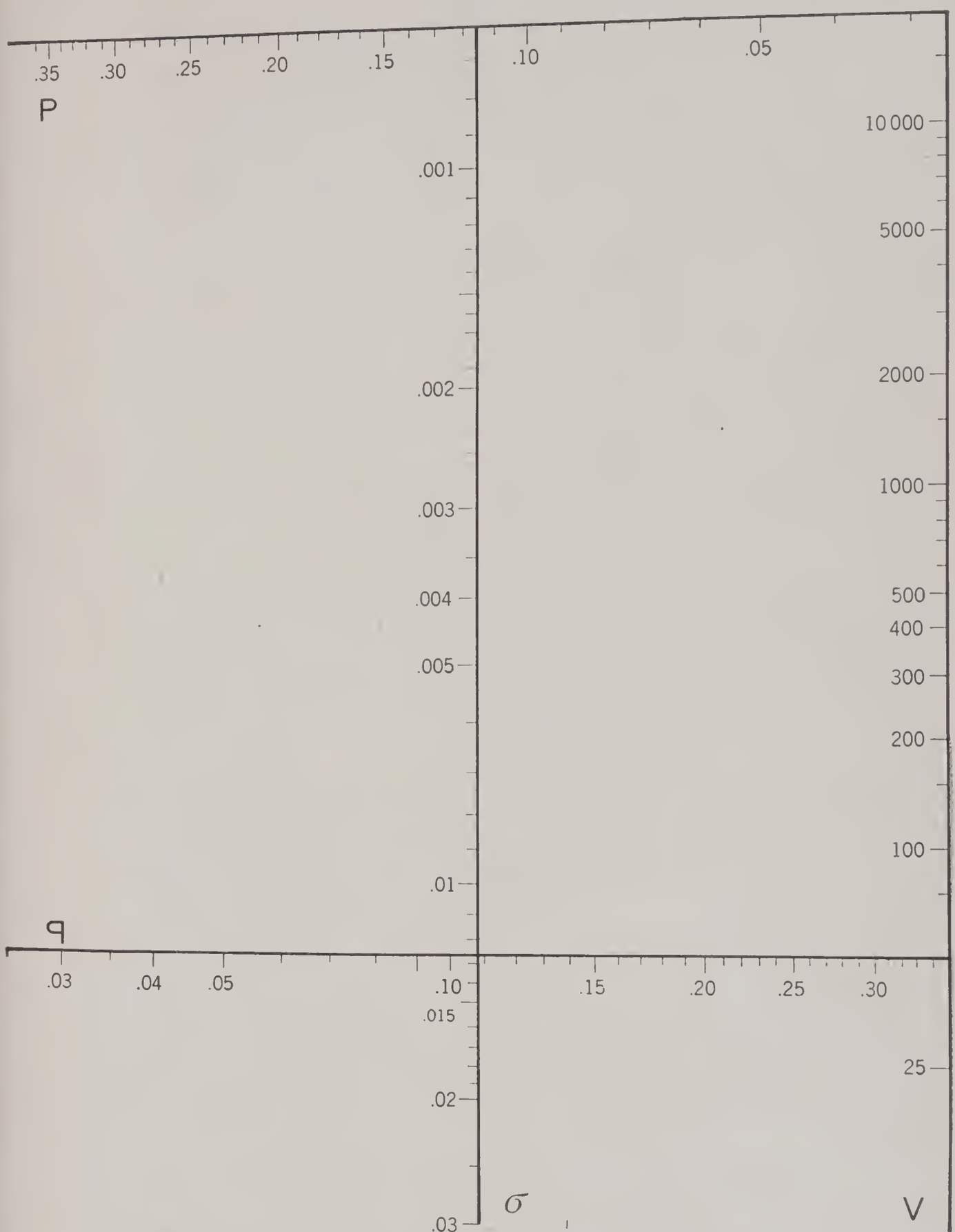

Fig. 42. Nomogram to compute standard deviation (σ) of $D = (p + q + r) - 1$. Use indicator (such as celluloid film) ruled with parallel and perpendicular lines. Set a line to cross scales P and Q at proper values, and a perpendicular to cross V at the number examined. This perpendicular will then cross scale σ at the proper value.

He used a system of co-ordinates intersecting at angles of 60°. The values of p, q, and r at the point of intersection must add up to 1 (or 100), so that any point on the graph then represents three values such that p + q + r = 1. For an example, see figure 43, from a paper by Haldane (28).

If the differences between the two series is small, we should be cautious about drawing any conclusions. There is still the possibility that non-random sampling might have influenced the gene frequencies.

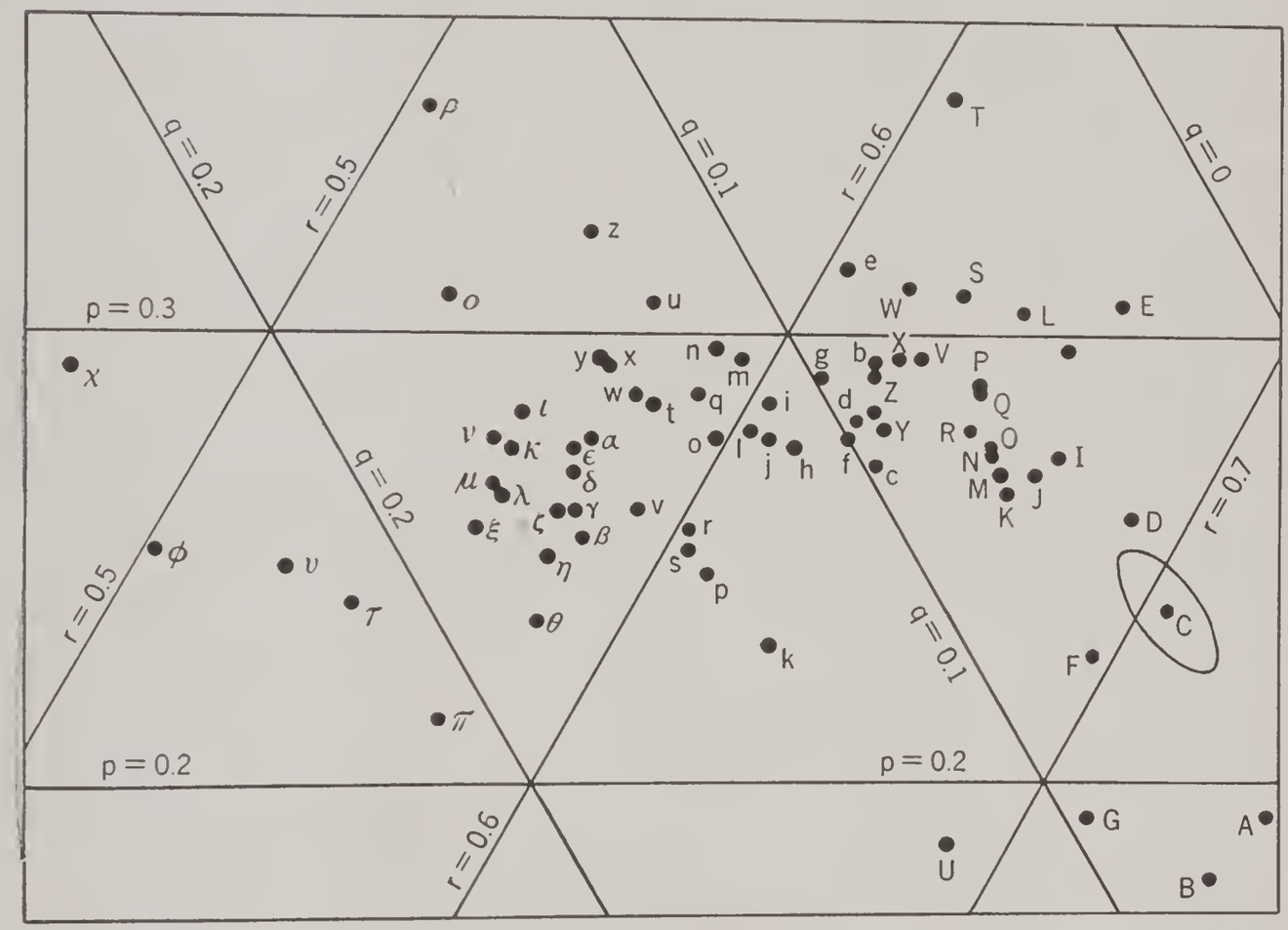

Fig. 43. Representation of blood grouping data in triangular coördinates (after Haldane, in *Human Biology*, **12**, 457, 1940). Key to symbols in original paper.

3.6515 Cartographic Representation. Cartographic representation of blood group distributions is subject to the same rules as other anthropological maps. First a map is made by plotting the values of each of the blood group genes, writing in the numerical values at the points (regions or centers) where the observations were made. The values can be divided into classes and different classes represented by suitable shading or differences in color. Suitable symbols for various gene frequencies may be placed at each point of observation. Figure 40, illustrating the M, N results, is an example of this. Streng (53) has proposed a single colored map showing the differences in p, q, and r frequencies by isogenes and the Ostwald color system.

Writing the values on whole areas such as political districts, instead of on *points* of observation is not free from objection, for it may produce the impression that observations are available for regions which in reality are

wholly uninvestigated, and the arbitrary character of the administrative or other boundaries may produce a distorted picture (5). If the districts are small enough, however, the result may be acceptable.

One of the above objections, namely that the impression may be produced that data are available for regions still unstudied, applies to some extent also to the construction of isogenes (lines through points supposed to have the same frequency of the gene being studied). But for many parts of the world the blood group data are now sufficiently numerous that isogenes are not too misleading, and they give rather a clear picture of the situation (see figures 38a and 38b).

3.6516 The Blood Characteristics A_1 and A_2, and M and N. Geographical variations in these gene frequencies have been established (7). It is not very difficult technically to add determinations of A_1 and A_2 to those for the classical groups (sec. 2.52). The requisite sera are, however, not too stable under transportation.

M and N determinations should always be carried out when sera can be obtained (sec. 2.4411).

For the entire blood group determination, then, four rows of tubes will be needed, instead of the two rows which suffice for determining the four classical groups. For all four of the rows immune sera can be used. Including controls, the entire set-up will look something like the accompanying example.

Row number	Serum	Control bloods	Bloods to be tested
1	Immune anti-B	O A_1 A_2 B	1 2 3 ....
2	Immune anti-A	O A_1 A_2 B	1 2 3 ....
3	Immune anti-M	M N MN	1 2 3 ....
4	Immune anti-N	M N MN	1 2 3 ....

The A bloods are then retested, using anti-A_1 and if possible, anti-A_2 serum.

Blood for the controls is to be taken when possible from persons whose blood characteristics have been carefully established previously (e.g. members of the expedition). If it is not possible to have all the desired controls during the actual investigations, some of the sera used should be brought back to the laboratory at the end and tested there against control bloods.

In representing the results, the following formulas may be found helpful ($\bar{O}$, $\overline{MN}$, etc. in these formulas represent the frequencies (per cent divided by 100) of the group in the population):

1. Frequency of gene for $A_1 = p_1 = \sqrt{\bar{O} + \bar{A}_1 + \bar{A}_2} - \sqrt{\bar{O} + \bar{A}_2}$
   Frequency of gene for $A_2 = p_2 = \sqrt{\bar{O} + \bar{A}_2} - \sqrt{\bar{O}}$
2. Frequency of gene for $M = m = \bar{M} + \overline{MN}/2$
   Frequency of gene for $N = n = \bar{N} + \overline{MN}/2$
   $D = 1 - (\sqrt{\bar{M}} + \sqrt{\bar{N}})$; $\sigma_D = 0.5/\sqrt{V}$ (See figure 44)

The ratio of the frequency of the two subgroups of A may be listed either directly as A_1/A_2 (7), or as the proportion of the genes, p_1/p_2 (44).

Since m increases in direct proportion to the decrease of n, i.e., $m + n = 1$, it is sufficient to present the frequency of one of the genes

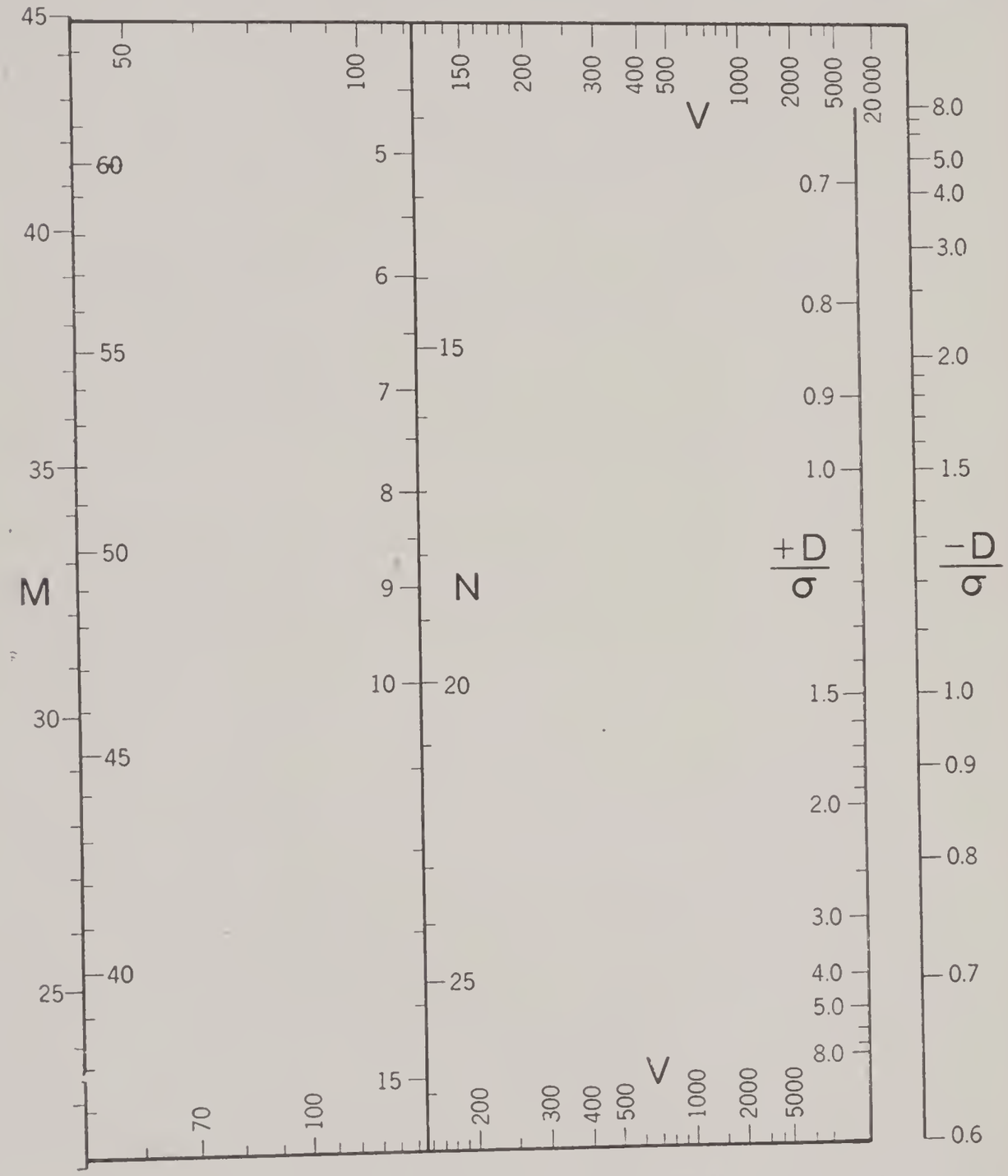

Fig. 44. Nomogram for testing internal consistency of M, N data. The nomogram computes $D/\sigma = [(m + n) - 1]/\sigma$. It is used similarly to figure 42. V and D/σ must be read on *connecting* scales, choosing one or the other as m + n is greater or less and 1.

only. Because of this simple relation a coordinate system is unnecessary to represent the results. The representation analogous to Streng's has been proposed by Mustakallio (44). All possible m, n combinations lie on a straight line, all possible M, N combinations on a curve.

Random variations in group frequencies will decrease, if the work is accurate, as the number investigated increases. An example from an actual investigation is shown in figure 45.

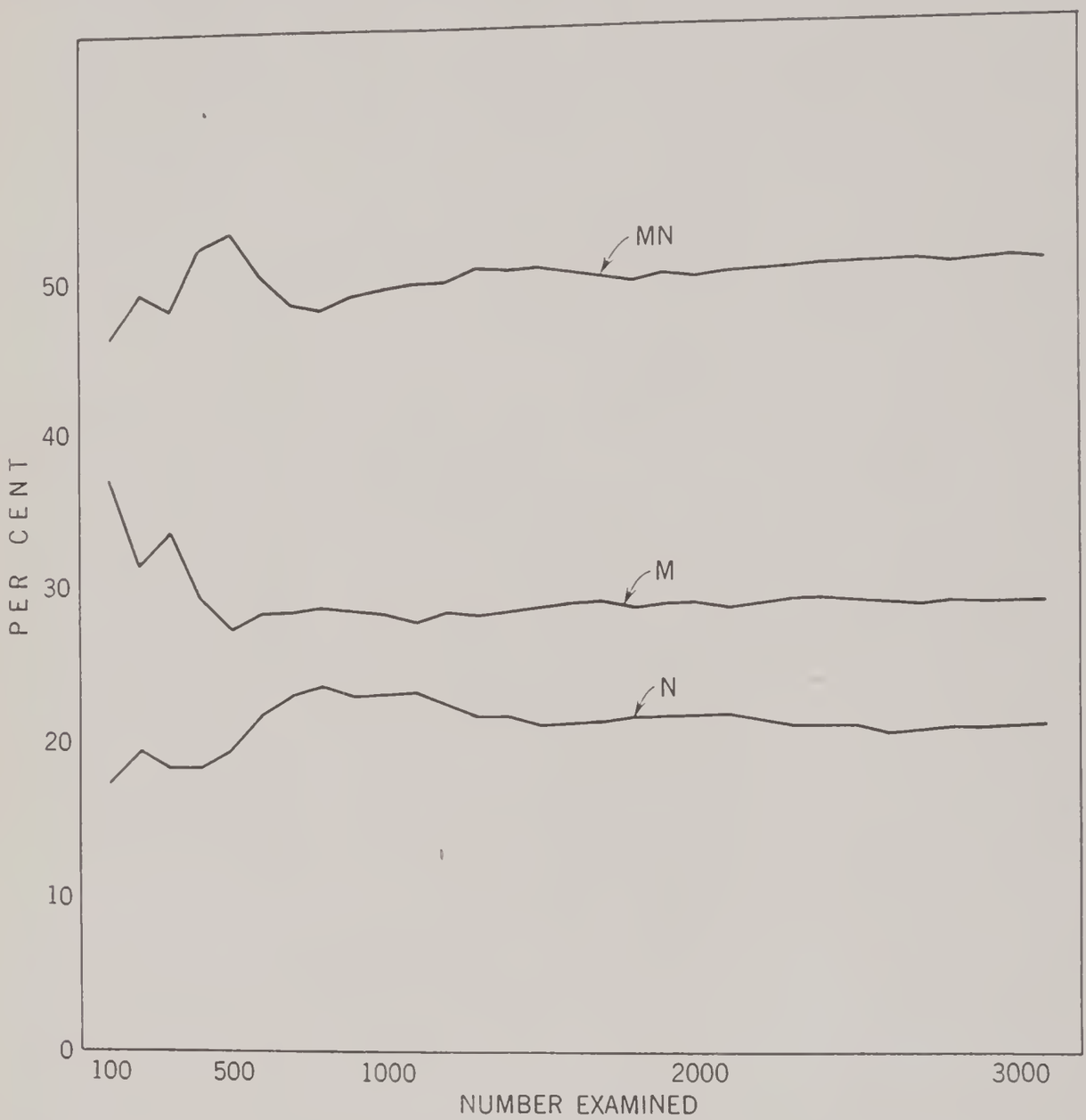

Fig. 45. Instance of random variations in type frequencies in populations of various sizes (Moureau).

3.6517 "Biochemical Index". It has been the custom of some authors to calculate for each population an "index" from the blood group frequencies (29). Some of these "indices" are:

"Biochemical race index" (29)	(A + AB)/(B + AB)
"Serological group index" (37)	(O + A)/(O + B)
"Biological race index" (from Bernstein's formulas)	= p/q
"Serological regression index" (54)	(A + AB)(O + B)/(B + AB)(O + A)
"Serological race index" (36)	group A/group B
"New biochemical race index" (41)	(O + A)/(B + AB)

It is not possible however, for a single ratio to indicate all the possible variations in three values capable of varying independently. Thus, the practical value of these indices is slight, and they will not be used here (55, 49).

3.6518 Publication of Data. The value of important data is sometimes impaired by improper presentation in the final publication. For the data to become fully available to other workers in the field, certain conditions must be fulfilled.

Full and complete details must be given concerning the ethnic group tested, where they live, and how the samples were selected. For instance, the designation "Arabs" is not nearly so informative as "Moslems, Baghdad, (patients in Royal Hospital)".

If several ethnic groups, possibly different, are represented in one locality, data for each group should be presented separately, followed by a general summary. This applies to different religions, etc.

In journals in which plenty of space is available, the actual numbers, as well as percents, in each group should be given. If this is not possible, the percents given should be checked carefully. Their sum should not differ from unity by more than 1 in the last decimal place. A surprising number of authors have published data containing statements like that of Babacan "who makes the truly remarkable assertion that of 100 Turks examined at Ankara, 9.3 per cent belong to group B" (cf. 28).

If data are presented which include previous results, of the author or of others, that fact should be clearly stated. The new data should be presented separately, as well as in summary.

3.652 Investigations on Archaeological Material

3.6521 General. In view of what has been said in section 3.645, it will be appreciated that methods of establishing the presence of certain blood groups among populations of the past are of considerable importance. This problem, which might have seemed hopeless, is seen on reflection not to be absolutely insoluble, in some cases.

The agglutinogens A and B are remarkable among antigens for their stability. Heating to 100°, drying, acids, mercuric chloride, formaldehyde,—none of these destroy A and B. As we have seen above, blood stains have been grouped after being dried for years. These and similar considerations suggested attempts to determine the blood groups of ancient, mummified human remains (12). The results suggest that the blood group picture in ancient peoples who left remains either accidentally or deliberately mummified, or skeletal material in a suitable state of preservation (17), can be approximately established.

3.6522 Mummified Tissue. Tests on dried material from necropsies show that the agglutinogens A and B are still detectable in recently dried muscle (sec. 3.421). The technic used is essentially similar to the methods used for dried blood stains (sec. 3.41322). The extension of the technic to mummified tissue (10, 11, 12, 13, 39, 19) was suggested by the hope that information thus obtained would throw light on the problem of the origin of the human blood groups, and might be of value to anthropologists and archaeologists.

Reagents. Not all A sera are found to have high enough titers. The B serum is diluted to make it of equal strength with the A. The A and B sera are not usually mixed for the test, on account of the possibility of some neutralization of the agglutinins by A and B substances normally occurring in the sera themselves. Type O serum is not used because of the commonly unequal strength of its agglutinins, and the possibility that the removal of one agglutinin from an O serum may in some cases involve some removal of the other (sec. 2.7221).

Immune sera, anti-A and anti-B (sec. 2.3) are also used, since they provide a valuable control on the specificity of the absorption (sec. 2.72).

3.65221 Procedure. The dried muscle tissue is ground fine in a mortar, and about 0.08 g. of it treated in a small test tube with 0.3 ml. of A serum which has been diluted, if necessary, so that further dilution of 1:8 gives a weakly agglutinating mixture, and dilution to 1:16 abolishes agglutination (cf. sec. 3.41322). Similar samples are also treated with diluted B serum, and with a mixture of anti-B and anti-A immune sera, diluted so that the mixture, diluted 1:8, agglutinates both A and B erythrocytes weakly, and, diluted 1:16, agglutinates neither. The resulting mixtures are thoroughly stirred and mashed with a stirring rod, left in the ice box 24 hours, mixed and mashed again, and at the end of 48 hours, the supernatants are removed and tested against standard A and B cells by the hanging drop method. For removal of the supernatants, as free as possible from debris, use may be made of a rubber bulb and a little pipette drawn out of glass tubing, with a wisp of cotton stuffed in its tip.

Failure of the supernatant to agglutinate standard A or B cells is taken to indicate removal of the corresponding agglutinin (cf. table 43a).

Samples which show removal of either or both agglutinins, from either the human or immune sera, or (as is usually the case) from both, plus a few which remove neither, for controls, should be again examined, by titration. Human sera of groups A and B, and a mixture of anti-A and anti-B sera, are used in volumes of 0.4 ml.; the sera should be somewhat stronger, having a titer of between 1:8 and 1:32. After the usual mixing and 48 hours standing, the supernatants are removed and titrated, using 0.1 ml. pipettes to make the progressively doubled dilutions.

Final decision as to the group of the mummy is based on the amount of the agglutinin removed, as judged from the titer before and after absorption. The serum should be checked from time to time for changes in strength. Most mummies either reduce the titer one tube, or do not affect it at all, but some specimens reduce it by several tubes. If the titer of a serum is reduced 3 tubes or more, under these conditions, it may be considered pre-

TABLE 64

TYPICAL RESULTS OF TESTS FOR ANTI-A AND ANTI-B AGGLUTININS IN SUPERNATANTS FROM MIXTURES OF MUMMY-TISSUES AND HUMAN, OR IMMUNE, SERA (13)

Number	Preliminary test				Titrations																	Group
					Immune serum										Human serum							
	Immune serum; cells		Human serum; cells		A cells					B cells					A cells					B cells					
					Dilutions, 1:																	
	A	B	A	B	2	4	8	16	32	2	4	8	16	32	2	4	8	16	32	2	4	8	16	32	
E60	+	+	+	−	+	+	±	−		±	−	−	−													B
E76	+	−	+	+	+	+	+	−		±	−	−	−		+	+	+	−		−	−					B
E100	±	±	−	−	+	+	+	−	−	+	+	+	+	−	+	+	−	−	−	+	+					A
E51	−	−	−	−	±	−	−	−		±	−	−	÷		−	−	−			−	−					AB
E52	+	+	+	+	+	+	+	+		+	+	+	÷		+	+	+			+	+					"O"
E56	+	+	+	+	+	+	+	−		+	+	+	÷		+	+	−			+	−					"O"
E39	−	−	+	+	+	+	+	±		+	+	±	−		+	+	+	+		+	+	+	+	−		"O"
A56	+	+	−	+	+	+	+	−	−	+	+	+	+	−	+	±	−	−	−	+	+					A
A140	+	±			+	+	+	−		±	−	−	÷							±	−	−	−	−		B
A146	+	−			+	+	+	−		+	+	−	−		+	+	+	±		+	−	−				B
A163	−	+	−	+	+	−	−	−		+	+	−	÷		±	−	−	÷								A
A78	+	+	+	+	+	+	+	+	−	+	+	+	+	±	+	+	+	+	±	+	+	+	−			"O"

Titers after absorption are given to the same number of stages as the original titer of the serum, to show degree of removal of agglutinin, since sera of different strengths were employed at different times. The symbol − indicates no agglutination. The symbol ÷ indicates that the serum before absorption gave only a ± reaction in this dilution, indicating that the appearance of a − reaction after absorption has less significance than if the original serum had here given a + reaction. The symbol "O" is used because no evidence exists to distinguish between the specimens really belonging to group O and those originally of other groups which have lost their absorptive power.

sumptive evidence of the presence of the appropriate agglutinogen in the mummified tissue. If the human and immune sera behave alike in this respect, as they usually do, the mummy is classified as A or B, as the case may be. If they do not behave alike, the mummy is retested, if sufficient material is available, or decision as to group withheld.

Failure of a tissue to remove either anti-A or anti-B agglutinin does not

necessarily mean that the individual belonged to group O. There is always the possibility that one or both of these agglutinins was originally present, but has disappeared or lost its reactive power, due to deterioration. Matson (39, 40) found that an absorbed bovine serum, which exhibited specific agglutination with O and A_2 cells, had its agglutinin removed by muscle and other tissue of group O, and not by group A. Boyd and Boyd, however, were not able to observe specific absorption of anti-O by muscle.

3.65222 Controls. (a) Dried human muscle of known group should be used as controls for the procedures employed for mummies. (b) When a tissue removes both anti-A and anti-B agglutinins, making it a pre-

TABLE 65

INDICATED BLOOD GROUPS OF MUMMIES OF VARIOUS ORIGINS

Number of specimens

Egyptians							Americans						
Period	Total	"O"	A	B	AB	Undecided	Period	Total	"O"	A	B	AB	Undecided
Predynastic	6	3	0	1	2	0	Pueblo I	1	1	0	0	0	0
Old Kingdom (I–VI dyn.)	7	4	0	1	1	1	Pueblo III	21	19	0	0	0	2
							Pueblo IV	2	2	0	0	0	0
Middle Kingdom (XI–XII)	2	1	1	0	0	0	Basket Maker	23	20	3	0	0	0
							Big Bend Basket Maker	8	7	0	1	0	0
New Empire (XVIII–XXIII)	23	15	2	2	2	2	Peruvian	134	118	1	6	2	7
Later material	74	62	0	7	4	1	Alaskan	4	4	0	0	0	0
Unknown	10	8	0	1	0	1	Others (or unknown)	12	12	0	0	0	0
Total.....	122	93	3	12	9	5	Total	205	183	4	7	2	9

sumptive "AB", test it with a diluted agglutinating serum for chicken or other unrelated erythrocytes, and titrate the supernatant, as above, against the chicken (or other) erythrocytes.

3.65223 Results. Certain samples of mummy tissue manifest a specific absorptive power for agglutinins anti-A and anti-B, *whether they are of human or immune animal origin.* Typical results are shown in table 64. Boyd and Boyd (13) examined in this way more than 300 mummies, American and Egyptian. Matson detailed tests on 23 mummies. In several samples a specific absorptive power indicated the presence of A and B. Such results were obtained with certain Basket Maker and Peruvian mummies; and in particular, among the Egyptians, A and B seem to be present in certain predynastic mummies more than 5000 years old (table 65).

Since the factors M and N have not proved to be demonstrable in normal muscle (sec. 3.4211), the M and N types in early peoples cannot be determined, at least by this technic.

3.6523 Bones.

3.65231 General. Candela has successfully modified the above technic so as to be able to determine the blood groups of dried bones (17, 19, 20). He has tested his technic by correctly grouping nine specimens, identified only by numbers, sent him by Boyd and Boyd. These bones came from human beings of known blood groups, and had been treated in various ways, as by drying in air, drying in the desiccator, and burial in earth. His correct grouping of the bones is shown in table 66.

3.65232 Directions for the Obtaining of Bone Samples for Blood Group Tests (after Candela, 18).

A. *Material.* (a) Prehistoric horizons are preferable, so that the possibility of admixture is avoided. (b) Material should be of known racial origins, or at least tentatively labelled. (c) The specimens selected should all be of the same culture level, and when possible, from the same or associated sites.

B. *Samples.* These can be prepared at the time of accession, and stored until tested. (a) Bones which are to be used for blood group tests should not be washed, but instead cleaned by brushing, or any dry method. (b) No preservatives should be used. Bones which have already been treated with ambroid, shellac or polymerized vinyl acetate may nevertheless be utilized provided the preservative has not penetrated into the cancellous portion of the bone. (c) Only intact bones should be used as a source of samples. (d) It is preferable, but not mandatory, that bones be not too badly weathered, as evidenced by marked porosity. (e) As far as possible, bones should be chosen from individuals whose cranial index is available. (f) The *ends* of the long bones, or if these are needed for morphological studies, the lumbar vertebrae are the best sources of cancellous bone. A sample may be made up of bone from various sites in the skeleton, provided, of course, that bones from more than one individual are not accidentally included. (g) The surface of the bone should be clean and dry; the instrument used should be wiped clean with alcohol and dried before each boring. (h) An ordinary bone curette can be used to scrape out the cancellous bone from the interior of the bone selected. The dense cortical bone is *not* to be included in the sample. (i) Each sample is put into a 1 ounce medicine vial having a screw cap. Enough cancellous bone should be taken to fill the bottle at least two-thirds full so that tests can be repeated when necessary. (j) The bottle should be sealed by means of non-porous adhesive tape, or by melted wax, to prevent

deterioration of the cancellous bone through the action of air. The identifying label should be distinct.

C. *Data.* These should be available, but may be withheld until tests are completed. (a), sex of each specimen, (b), cranial index of each specimen, (c), information as to racial connections and culture elements.

3.65233 Technic of Tests. Candela uses A and B sera for this work, diluted so as to have titers of about 1:16. The titers are determined by a micro "slide" technic, as follows: successively doubled serum dilutions are prepared in the usual way. From each tube 0.5 cc. is measured accurately with a syringe into the well of a "well slide" (sec. 2.1). There is added 0.05 cc. of a 1 per cent suspension of test cells. The slides are agitated at frequent intervals and the final readings made under the microscope at the end of 30 minutes. Candela states (21) that the results are the same if cells and serum are mixed, *in the same proportion*, in 7 mm. tubes, which are centrifuged after standing 30 minutes.

This method of titration gives titers about 8 times as high for a given serum as would be obtained by a "test tube" method (mixing a 2 per cent cell suspension directly with an equal volume of diluted serum), and is an important feature of the method. The increased sensitivity resulting from the use of lower final concentrations of erythrocytes in agglutination reactions has also been pointed out by Lund (38).

Outline of procedure. We give this substantially in Candela's own words (19). It is offered as a guide in developing a working routine, and is not to be taken as a rigid model to be followed under all conditions. No such model is possible, in view of the variables which may be encountered. For instance, the amounts of ground-up bone, and consequently of test sera, represent merely the quantities conveniently handled and designed, above all, to result in successful tests with the material at hand. These amounts may be varied as conditions require.

The factors with which no compromise is possible are: first, equality in the agglutinative powers of the sera, and in the amounts of A and B sera used in each test; second, equality in amounts of ground up bone in the two tubes; third, the use of a sufficiently wide range of dilutions, and fourth, careful controls of the reactions obtained.

Pulverize one-half or less of the specimen in a mortar which is cleaned and dried each time before using. Discard irreducible fragments by sifting.

Using dry, sterile syringes and needles, test the A and B sera which are to be used in the typing of the specimens (serum control).

With a small graduate, measure 0.25 cc. of ground-up bone into each of two dry, sterile test tubes. To one add 0.9 cc. of A test serum, and to the other an equal amount of B test serum. Avoid splashing against the

side of the tube. Close the tube with a dry, sterile rubber cap, and then, holding the tube in one hand at an angle of 45°, rotate it carefully, meanwhile flicking it with the fingers of the other hand until thorough mixing has occurred. This may take some time, but is of the utmost importance.

At each testing, known A and B specimens should be set up in the same manner, preferably specimens from the same series. This affords an opportunity of repeating the tests several times with each specimen (specimen controls).

When all the specimens, known and unknown, have been set up, they are placed in the refrigerator, together with the remaining test sera. They should be agitated as described at intervals of about 12 hours, and removed from the refrigerator at the end of 48 hours.

When the specimens and the test sera have come to room temperature, the A and B sera are again tested to detect changes in their agglutinative powers. The erythrocyte suspensions are made by first allowing a few drops of blood to fall into a 1% sodium citrate solution in normal saline, and then adjusting them so that they are approximately equal. This is done by centrifuging the suspensions to drive down the cells, then preparing new suspensions by using measured amounts of the erythrocyte sediment.

After the test sera have been checked, and any changes in their strengths noted, the specimen tubes are centrifuged. The supernatant sera of each are drawn up, using a syringe with a long needle so that the amounts of serum taken up from the A and B tubes are equal. These are tested against the same erythrocyte suspensions which were used in checking the test sera. Either small test tubes or glass slides may be used, but in either case the progress of agglutination must be followed under the microscope, so that comparisons can be made in the speed of agglutination, and the size of the clumps in the two sera of each specimen, and among the various specimens themselves. The same procedure is followed with the controls.

Candela later indicates his preference for using the above "micro" slide technic for the tests.

It is often found that the fluid has become hemolytic for erythrocytes, due (a) to presence of acid, or (b) to presence of glycerol. If hemolysis occurs, the resulting supernatant is tested with litmus paper or methyl red, and if acid, neutralized by the addition of weak (N/50) alkali, drop by drop. If it is not acid, a small amount of a saturated solution of NaCl is added. This was sometimes found sufficient for the acidic supernatants.

To the residues of those specimens which gave A, B, or AB reactions, another 0.9 cc. of test serum is added. This is done to determine whether the samples are capable of adsorbing still more agglutinins (reinforced reaction). These are replaced in the refrigerator for an additional 48 hours of agitation and incubation.

Those specimens which have not cleared either test serum of its agglutinins are re-tested. This time, however, fresh bone samples are immersed in sera which are one half the previous strengths. Occasionally this re-testing may bring out an A or a B reaction which was too weak to be revealed at the stronger dilution level. If, however, as is usually the case, residual agglutinins are again found in both tubes, the specimen is considered to be group O.

3.65234 Interpretation. If the test sera have been shown to be of equal powers at the time of setting up the tests, and again at the actual testing 2 days later, the following conclusions may be drawn: a) all specimens

TABLE 66

RESULTS OF TESTS PERFORMED BY CANDELA ON UNKNOWN SPECIMENS OF BONES FROM PERSONS OF KNOWN BLOOD GROUP

Number	Method of aging	Hemolytic effect	Due to	Known group	Reported group	Number of reinforced reactions
C-18	Air	Slight	Acid	A	A	3
C-19	Air	Marked	Acid	O	O	
C-20	?	Marked	Glyc.	O	O	
C-21	Des.	Marked	Both	A	A	10
C-22	Air	None		A	A	2
C-23	Des.	Marked	Both	B	B	7
C-24	Bur.	None		B	*	4
C-25	Air	None		O	O	
C-26					**	
C-27	Des.	None		B	B	9
C-28	Air	Slight	Acid	B	B	7

des. = desiccator; bur. = buried.

* No report made, as reaction did not meet requirement of section 3.65234 (c).

** Cortical bone only, unsuitable for testing.

giving a clear-cut differential A or B reaction may be considered to be of the group indicated, even when a reinforced reaction is not obtained. Such specimens, however, should be given repeated tests, by using them as specimen controls. b) All specimens which failed to clear the agglutinins from sera one-half as strong as the norm for the series are best considered to be group O, especially if the amounts of residual agglutinins in the two tubes are equal. If there should be a consistent, appreciable difference, however, the specimen may be a badly deteriorated A or B, and should be investigated further. c) Those specimens which cleared agglutinins from both tubes at the "normal" dilution of sera, and also gave a reinforced reaction, may safely be considered to be group AB, provided that they have also

fulfilled the additional requirements that they be not isolated specimens, and that the remainder of the series contains at least one A and one B.

It will be noted that the diagnosis of group is made similarly to that of mummies, but even greater care must be taken to avoid errors due to non-specific removal of agglutinins. If only one agglutinin, say anti-A, is removed, the diagnosis is group A; if both are removed, the successive additions of serum are continued until removal of one agglutinin ceases, while that of the other continues. In such cases the removal of the first agglutinin is considered to be due to a non-specific reactivity of the material, and the significant part of the test begins after this has ceased.

The criteria for diagnosing a group AB (see above, (c)), perhaps deserve special emphasis.

The results of Candela's tests on the specimens sent him by Boyd and Boyd are shown in table 66.

3.65235 Precautions. In work as delicate as this, the use of proper controls and precautions is of the greatest importance. The following factors operate to render the technic somewhat complicated: (a) inequalities in the agglutinative powers of A and B test sera; (b) inequalities in the powers of the various bone specimens, even those in the same series, to adsorb agglutinins from test sera; (c) deterioration of the A and B test sera, with the loss of their agglutinins, often proceeding at an unequal rate; (d) deterioration of the ground-up bone due to the enormous increase in the amount of surface exposed to the air; (e) non-specific adsorption of both α and β agglutinins from test sera by ground-up bone; (f) the impossibility of distinguishing an O specimen from one which has lost its agglutinogens by deterioration; (g) the necessity for rigid standards of identification of the presumptive AB specimens. These are necessary because under certain conditions, the distinction between O and AB rests primarily upon the strength of the sera which are used in testing a bone specimen. It would be a serious mistake to report a false AB, since in so doing, both the A and the B factors would erroneously be ascribed to a population which may have lacked one of them or both. To report group O in a specimen which actually contains A or B is of course a much less serious error, since "O" in these tests is used to denote a blank, and carries no implications.

Since the sera are so dilute, they deteriorate rapidly. They should be handled at all times under aseptic conditions. All test tubes, vials, rubber caps and syringes must be sterilized before they are used. The original sera and saline used in making the dilutions must contain a preservative. "Merthiolate" is recommended for this purpose, added in such quantities as to make a final dilution of 1:5000 or 1:10,000.

The serum and saline should be accurately measured, as for example by means of "tuberculin" syringes and needles. These may also be used for measuring the amounts of cell suspension used, or the size of the drop delivered by a hypodermic needle may be calibrated. Many 26 gauge hypodermic needles deliver almost exactly 100 drops to the cc., so that in such cases 5 drops will equal 0.05 cc., the amount usually required.

To allow for inequalities in the specific absorptive power of bone specimens, a range of serum dilutions is recommended, sufficiently wide to include, at one end, serum which will probably be too strong and at the other, serum which will probably be too weak.

All isolated specimens should be tested over the entire range. When a series of fairly uniform specimens is being studied, it is sufficient to test first 10 or 12 in this manner, until the dilutions of serum most favorable for A and B reactions are determined, then to use these dilutions as a

TABLE 67

CANDELA'S RESULTS ON ANCIENT BONE

Source of material	Age	Number of samples	Group			
			O	A	B	AB
Egypt	1500 B.C.	11	2		6	3
Aleutian islands	Pre-Russian + early Russian	30	11	11	6	2
Minnesota	?	6	1	5		
New York	ca. 1600 A.D.	4			4	

working norm. Specimens giving "O" or "AB" reactions, however, must be investigated further. The former are tested with weaker dilutions, noting in each test whether the residual agglutinins appear to be equal in amount. The AB case has already been discussed.

3.65236 Results. Results obtained by Candela on application of his technic to ancient bone are shown in table 67.

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